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1 Manuscripts should be sufficiently detailed to permit a critical appraisal of the work. Conclusions should be based upon the experimental data submitted

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SECTION MEETINGS

NEW YORK

New York Academy of Medicine

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ROCKY MOUNTAIN

University of Colorado

December 7, 1945

MINNESOTA

University of Minnesota

December 19, 1945

15206

A Simple Method for the Concentration of Rh Agglutinins

ERNEST WITEBSKY, JAMES F MOHN, DORIS J HOWLES, AND HELEN M WARD

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and the Buffalo General Hospital*

There are two sources of human anti-Rh serum. Following multiple transfusions of Rh-positive blood certain Rh-negative individuals develop Rh antibodies¹. The more common source of human Rh antibodies, however, is found in women who have given birth to erythroblastotic children². Rh agglutinins if present are frequently of low titer. The procedure for fractionated precipitation of sera containing antibodies has been developed especially in Dr. Cohn's laboratory at Harvard and is successfully applied in the case of anti-Rh serum (Diamond). However, this method requires special equipment and training and is not available to everybody. It seemed to be desirable to find means and ways to concentrate Rh agglutinins by a simple method.

Anti-Rh sera were subjected to dialysis in

the following manner. The native serum is placed in a cellophane tubing bag under tension, air having been eliminated as much as possible. The bag is then suspended in a battery jar containing a large volume of freshly distilled water. The serum is dialyzed 24 to 30 hours in the icebox at 4°C, the distilled water being changed several times during the procedure. During dialysis at this temperature a precipitate forms. The serum with its precipitate is removed from the cellophane bag and the precipitate is separated from the supernatant fluid by centrifugation. In the experiments to be described, the sediment (precipitate) is dissolved in saline solution and referred to as the globulin fraction. The globulin fraction obtained from 10 cc of each serum is dissolved in 1 cc of saline solution (0.9% NaCl) by vigorous stirring, then kept for 2-3 hours in the icebox. Insoluble particles are removed by centrifugation. The dissolved globulin is preserved in the frozen state at -20°C or

1 Wiener, A. S. and Peters, H. R. *Ann. Int. Med.*, 1940, 13, 2306.

2 Levine, Philip. *Am. J. Obs. and Gyn.* 1945, 49, 810.

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There are two sources of human anti-Rh serum. Following multiple transfusions of Rh-positive blood certain Rh-negative individuals develop Rh antibodies¹. The more common source of human Rh antibodies, however, is found in women who have given birth to erythroblastotic children². Rh agglutinins if present are frequently of low titer. The procedure for fractionated precipitation of sera containing antibodies has been developed especially in Dr. Cohn's laboratory at Harvard and is successfully applied in the case of anti-Rh serum (Diamond). However, this method requires special equipment and training and is not available to every body. It seemed to be desirable to find means and ways to concentrate Rh agglutinins by a simple method.

Anti-Rh sera were subjected to dialysis in

the following manner. The native serum is placed in a cellophane tubing bag under tension, air having been eliminated as much as possible. The bag is then suspended in a battery jar containing a large volume of freshly distilled water. The serum is dialyzed 24 to 30 hours in the icebox at 4°C, the distilled water being changed several times during the procedure. During dialysis at this temperature a precipitate forms. The serum with its precipitate is removed from the cellophane bag and the precipitate is separated from the supernatant fluid by centrifugation. In the experiments to be described, the sediment (precipitate) is dissolved in saline solution and referred to as the globulin fraction. The globulin fraction obtained from 10 cc of each serum is dissolved in 1 cc of saline solution (0.9% NaCl) by vigorous stirring, then kept for 2-3 hours in the icebox. Insoluble particles are removed by centrifugation. The dissolved globulin is preserved in the frozen state at -20°C or

¹ Wiener, A. S. and Peters, H. B. *Ann. Int. Med.* 1940, 13: 2306.

² Levine, Philip. *Am. J. Obs. and Gyn.* 1945, 49: S10.

TABLE I

Agglutination of Rh Positive Group O Cells by Serum (Dem)

Serum (Dem)			
	a	b	c
	Native	Supernatant	Globulin
1 Undiluted	++++	+++	++++
2 1 2	+++	++	++++
3 1 4	+++	+	++++
4 1 8	+++	+	++++
5 1 16	++	±	++++
6 1 32	++	—	++++
7 1 64	+	—	+++
8 1 128	±	—	+++
9 1 256	—	—	++
10 1 512	—	—	++
11 1 1024	—	—	±
12 Saline	—	—	—

— = No agglutination
 ± = Faint agglutination
 + = Slight agglutination
 ++ = Marked agglutination
 +++ = Strong agglutination
 ++++ = Very strong agglutination

lyophilized The supernatant fluid to which a sufficient amount of 10% saline solution is added to bring the final salt concentration to 0.9% is referred to in this paper as the "supernatant" In former years the described procedure served as a rough method to separate the globulin and the albumin fractions of serum It is now generally agreed that the albumin fraction obtained by dialysis is by no means pure but still contains some globulin

The analysis of several anti-Rh sera is reported in this paper One serum (Kru) was obtained from an Rh-negative patient who had received multiple transfusions and who had died after a long chronic illness A large amount of blood containing an Rh agglutinin of relatively weak potency was collected after the patient's death The second serum (Dem) and the third serum (Heng) were obtained from Rh-negative women who had given birth to erythroblastotic children, so was a fourth serum which contained "blocking antibodies" only⁵

The first experiment was carried out in the following way Decreasing amounts of serum (Dem) and its fractions respectively (volume 0.1 cc) were mixed with 0.1 cc of a 2% suspension of Rh-positive Group O cells After standing at room temperature for one hour the tubes were centrifuged at medium speed for 1 minute and read for agglutination

The experiment shows that the globulin

fraction, dissolved in one-tenth of the original volume of the serum, contains a potent Rh agglutinin Characteristic of the agglutination of Rh-positive cells by the globulin fraction is not only an increase in end titer but also an increase in the relative strength or quality of the agglutination showing a 4+ agglutination up to a dilution of 1:32 In contrast, the supernatant fluid is poor in Rh agglutinins when compared with the native untreated serum From this experiment the conclusion can be drawn that the Rh agglutinins are found in the "globulin" fraction as obtained by simple dialysis and only a minor portion remains in the supernatant fluid

The discovery of the "incomplete antibody" (Race^{3,4}) or the "blocking antibody" (Wiener⁵) in the serum of patients expected to contain Rh antibodies has proved to be of great importance This antibody uniting with Rh-positive cells, thus sensitizing them, will prevent agglutination of Rh-positive cells by the Rh agglutinin Diamond's and Abelson's⁶ investigations have shown that "blocking antibodies" when mixed with whole blood will lead to agglutination of Rh-positive cells on the slide as well as in the test tube As demonstrated by Diamond and his co-workers^{7,8} and Wiener,⁹ agglutination of Rh-positive cells by "blocking antibodies" can be easily obtained if undiluted serum or 20% albumin replaces saline solution as a diluent

The following experiment as recorded in Table II deals with serum (Kru) and its fractions derived by dialysis The experiment itself was carried out in two parts In the first part, all dilutions were made in saline solution, and for the second part all dilutions

³ Race, R. R., *Nature*, 1944, **153**, 771

⁴ Race, R. R., and Taylor, G. L., *Brit Med J*, 1944, **2**, 756

⁵ Wiener, A. S., *Proc Soc Exp Biol and Med*, 1944, **56**, 173

⁶ Diamond, L. K., and Abelson, N. M., *J Lab and Clin Med*, 1945, **30**, 204

⁷ Diamond, L. K., and Abelson, N. M., *J Lab and Clin Med*, 1945, **30**, 668

⁸ Diamond, L. K., and Denton, R. L., *J Lab and Clin Med*, 1945, **30**, 821

⁹ Wiener, A. S., *J Lab and Clin Med*, 1945, **30**, 662

TABLE II
Agglutination of Rh₁ Group O Cells in Serum (Kru)

Serum (Kru)	I. All dilutions in saline			II. All dilutions in serum		
	a	b	c	a	b	c
	Nature	Supernatant	Globulin	Nature	Supernatant	Globulin
1. Undiluted	---	---	---	---	---	---
2. 1:2	---	---	---	---	---	---
3. 1:4	---	---	---	---	---	---
4. 1:8	---	---	---	---	---	---
5. 1:16	---	---	---	---	---	---
6. 1:32	---	---	---	---	---	---
7. 1:64	---	---	---	---	---	---
8. 1:128	---	---	---	---	---	---
9. 1:256	---	---	---	---	---	---
10. 1:512	---	---	---	---	---	---
11. 1:1024	---	---	---	---	---	---
12. Saline	---	---	---	---	---	---

(cells serum and its fractions) were made in undiluted serum

Decreasing amounts of serum (Kru) and its fractions (volume 0.1 cc) were mixed with 0.1 cc of Rh₁ cells belonging to Group O. After standing for one hour at room temperature the tubes were centrifuged and read.

Experiment II again demonstrates the fact that Rh agglutinins appear in the globulin fraction. In this case the supernatant appears to be completely free of agglutinins. However when diluted in serum the supernatant reveals the presence of antibodies of the type referred to as blocking or incomplete antibodies. It should be pointed out that the untreated serum when diluted in serum does not reveal a higher antibody titer than when diluted in saline solution. One might therefore come to the conclusion that serum (Kru) is free of blocking antibodies but actually the presence of such antibodies can be demonstrated by splitting the serum into fractions by dialysis and using serum as a diluent instead of saline. The relative effectiveness of the globulin fraction in respect to the agglutination of Rh-positive cells in a saline medium can therefore be explained not only by concentration of the Rh agglutinins but also by the fact that the accompanying blocking antibodies have not been concentrated to the same extent. The same results as those reported in Table II were observed when Rh₂ and Rh₃ cells were used instead of Rh₁ cells.

The presence of blocking antibodies in

serum (Kru) manifests itself easily if a different order of experiment is used as shown in Experiment III.

Decreasing amounts of (a) serum obtained from an Rh-negative mother who had an erythroblastotic child and whose serum contained blocking antibodies only (b) a normal serum (as a control) (c) the supernatant fraction of serum (Kru) (volume 0.15 cc) were mixed with 0.05 cc of a 6% suspension of Rh₁ cells belonging to Group O. After incubation for 30 minutes in the water bath at 37°C 0.05 cc of undiluted anti-Rh serum (Diamond B-2X) were added. The tubes after being shaken well were kept for an additional hour at 37°C and centrifuged.

The blocking effect of the supernatant (Kru) which equals in strength the blocking property of an untreated serum of an erythroblastotic woman known to contain blocking antibodies is evident from this experiment. The question arose whether the incomplete or blocking antibody was to be found also in the globulin fraction or whether this antibody would remain in the supernatant following dialysis. For that reason serum (Heng) containing blocking antibodies exclusively was selected and subjected to dialysis using the technique as described above. The untreated serum as well as the two serum fractions obtained by dialysis were then examined in the following way.

Decreasing amounts of whole serum (Heng) and its serum fractions respectively (volume 0.1 cc) were mixed with 0.1 cc of a 2%

TABLE III
Inhibitory Effect of "Blocking Antibodies" on
Agglutination of Rh₁ Cells by Rh Agglutinins

Serum containing blocking antibodies	Blocking serum	Normal serum (control)	Serum (KRU) supernatant
1 Undiluted	—	+++	—
2 1:1	—	+++	—
3 1:2	—	+++	—
4 1:4	+	+++	±
5 1:8	++	+++	+
6 1:16	+++	+++	++
7 1:32	+++	+++	+++

suspension of Rh₁ cells belonging to Group O. The experiment was carried out in two parts. Serum dilutions and blood cell suspensions were made (I) with saline solution and (II) with undiluted normal human serum. Tubes were kept for one hour in a water bath at 37°C, and then centrifuged for one minute at a medium speed. The resulting agglutination is shown in Table IV.

The native serum (Heng) as well as the serum fractions obtained by dialysis do not give any visible agglutination with Rh₁ cells. Group O when diluted in saline solution. However, if undiluted normal serum is used as a diluent, agglutination of Rh-positive cells occurs in rather high dilutions. The fraction referred to as "supernatant" shows definite agglutination indicating that the "blocking antibody" is present almost to the same extent as in the untreated serum. The globulin frac-

tion also contains some 'blocking'. Considering the fact, however, that dealing here with an approximately 1 concentrated fraction it becomes obvious a large portion of the 'blocking' has remained in solution (supernatant) during dialysis.

The serum fractions obtained by dialysis were also examined for the presence of agglutinins anti-A and anti-B. Some unusual results were obtained. The result was that dialysis would divide the agglutinins more or less equally between the globulin fraction and the supernatant. In many instances the supernatant contained more isoagglutinins anti-A than the globulin fraction.

Conclusions The dialysis of serum containing Rh antibodies permits the division of such a serum into two fractions, the supernatant, containing mostly globulins, and a certain amount of the globulin. The sediment, when dissolved in physiological saline solution using one-tenth of the volume of the serum prior to treatment, shown to contain the major portion of the agglutinins. A rather potent Rh test can be obtained by this procedure, in many instances anti-Rh sera containing agglutinins of such weak potency that

TABLE IV
Titration of Rh "Blocking Antibodies" in the Different Fractions of Serum (Heng) A Rh₁ Cells

	a Native		b Supernatant		c Globulin	
	I Diluted with saline	II Diluted with serum	I Diluted with saline	II Diluted with serum	I Diluted with saline	II Diluted with serum
1 Undiluted	—	++	—	—	—	+
2 1:2	—	++	—	+	—	+
3 1:4	—	+++	—	++	—	+
4 1:8	—	+++	—	+++	—	+
5 1:16	—	+++	—	+++	—	+
6 1:32	—	+++	—	+++	—	+
7 1:64	—	+++	—	+++	—	+
8 1:128	—	+	—	++	—	+
9 1:256	—	±	—	±	—	+
10 1:512	—	—	—	±	—	+
11 1:1024	—	—	—	—	—	+
12 Saline	—	—	—	—	—	—

could be strong enough to serve as fixation serum. The method is especially recommended for this purpose. The "class" or "blocking antibody" has been found to be associated to a large extent with globulin, indicating that at least a separation of the Rh agglutinin from "blocking antibody" has been accomplished by dialysis. The effectiveness of the

resulting Rh agglutination by the globulin fraction is therefore not only attributed to simple concentration of the Rh agglutinins but also to the partial removal of the Rh blocking antibody. The isoelectroglutinins anti-A and anti-B are not concentrated to the same extent as the Rh agglutinins which constitutes an additional advantage of the globulin preparation over the untreated serum.

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Results of Inoculating Okinawan Horses with the Virus of Japanese B Encephalitis

LEWIS THOMAS* AND JOHN L. PECK. (Introduced by T. M. Rivers)

From U. S. Naval Medical Research Unit No. 2.

has been reported previously¹ that a considerable percentage of Okinawan horses in which samples of serum were obtained during an epidemic of Japanese B encephalitis possessed antibodies against the virus responsible for the epidemic. This observation suggests that horses may occupy the role of intermediary hosts for the virus. The importance of this role in the epidemiology of the disease in human beings depends to a large extent upon whether the virus is capable of circulating in the blood stream of infected horses, from which source it might be transmitted to man by mosquitoes. In order to obtain information concerning the matter, four Okinawan horses were inoculated with the virus of Japanese B encephalitis on 16 September, 1945. The present report is concerned with the results of the experiment.

Materials and Methods. Through the cooperation of the Commander of Naval Construction Troops, a screened, wooden-frame

stable was built on the outskirts of the village of Ishikawa. This building contained four stalls, a separate room for laboratory studies and a concrete floor with drainage outlets leading to a nearby pit. Throughout the course of the experiment the interior was thoroughly sprayed each day with DDT.

Four horses were selected from the corral at Ishikawa. Like virtually all other horses on Okinawa these were malnourished, undersized animals whose general physical condition was obviously poor. Three of the horses were approximately one year old, the fourth was a Mongolian pony approximately 12 years old. Each horse was inoculated intravenously with 2 cc of a 10% suspension of mouse-brain tissue infected with a strain of Japanese B encephalitis virus isolated during this epidemic from a fatal case of encephalitis.² Specimens of blood were obtained from each horse before the injection of virus and at intervals of 16 hours, 3, 6, 9 and 12 days after inoculation. Neutralization tests were performed with the pre-inoculation sera in order to determine the status of immunity in each animal at the time of injection. The specimens of blood taken after inoculation were injected intracerebrally and intraperitoneally into mice in order to determine the presence or absence of virus. In the instances where a virus was recovered

* The ideas set forth herein are those of the authors and are not necessarily those of the Bureau of Medicine and Surgery of the Navy Department.

† The authors are indebted to Captain E. G. Euley, (VC), AUS, Assistant Veterinarian, 10th Army, for assistance in the handling and care of the horses during the experiment. Acknowledgment is also made to CPhM I. B. Stacey, USN, for technical assistance.

¹ Hodes, H. L., Thomas, L. and Peck, J. L., *Science*, in press.

² Hodes, H. L., Thomas, L. and Peck, J. L., *Proc. Soc. Exp. Biol. and Med.*, 1945, 60, 220.

TABLE III
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Serum containing blocking antibodies	a Blocking serum	b Normal serum control	c Serum (KRU) supernatant
1 Undiluted	—	+++	—
2 1 3	—	+++	—
3 1 9	+	+++	±
4 1 27	++	+++	+
5 1 81	+++	+++	++
6 0	+++	+++	+++

suspension of Rh₁ cells belonging to Group O. The experiment was carried out in two parts. Serum dilutions and blood cell suspensions were made (I) with saline solution and (II) with undiluted normal human serum. Tubes were kept for one hour in a water bath at 37°C, and then centrifuged for one minute at a medium speed. The resulting agglutination is shown in Table IV.

The native serum (Heng) as well as the serum fractions obtained by dialysis do not give any visible agglutination with Rh₁ cells Group O when diluted in saline solution. However, if undiluted normal serum is used as a diluent, agglutination of Rh-positive cells occurs in rather high dilutions. The fraction referred to as "supernatant" shows definite agglutination indicating that the "blocking antibody" is present almost to the same extent as in the untreated serum. The globulin frac-

tion also contains some "blocking antibody." Considering the fact, however, that we are dealing here with an approximately 10 times concentrated fraction it becomes obvious that a large portion of the "blocking antibody" has remained in solution (supernatant) following dialysis.

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Conclusions The dialysis of serum containing Rh antibodies permits the division of such a serum into two fractions, the precipitate, containing mostly globulins, and the supernatant, containing the albumin fraction and a certain amount of the globulin fraction. The sediment, when dissolved in physiological saline solution using one-tenth of the original volume of the serum prior to treatment, is shown to contain the major portion of the Rh agglutinins. A rather potent Rh testing serum can be obtained by this procedure, where in many instances anti-Rh sera contain agglutinins of such weak potency that the native

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Rh₁ Cells

	a Native		b Supernatant		c Globulin	
	I Diluted with saline	II Diluted with serum	I Diluted with saline	II Diluted with serum	I Diluted with saline	II Diluted with serum
1 Undiluted	—	+	—	+	—	++
2 1 2	—	++	—	++	—	+
3 1 4	—	+++	—	++	—	+
4 1 8	—	++	—	+++	—	+
5 1 16	—	++	—	+++	—	++
6 1 32	—	++	—	++	—	++
7 1 64	—	++	—	++	—	++
8 1 128	—	+	—	+	—	++
9 1 256	—	±	—	±	—	+
10 1 512	—	—	—	—	—	—
11 1 1024	—	—	—	—	—	—
12 Saline	—	—	—	—	—	—

serum would not be strong enough to serve as a diagnostic test serum. The method is especially recommended for this purpose. The "incomplete" or "blocking antibody" has been found to be associated to a large extent with the supernatant, indicating that at least a partial separation of the Rh agglutinin from the "blocking antibody" has been accomplished by dialysis. The effectiveness of the

resulting Rh agglutination by the globulin fraction is therefore not only attributed to simple concentration of the Rh agglutinins, but also to the partial removal of the Rh "blocking antibody". The isoagglutinins anti-A and anti-B are not concentrated to the same extent as the Rh agglutinins which constitutes an additional advantage of the globulin preparation over the untreated serum.

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LEWIS THOMAS*† AND JOHN L. PECK (Introduced by T. M. Rivers)

From U. S. Naval Medical Research Unit No. 2

It has been reported previously¹ that a considerable percentage of Okinawan horses, from which samples of serum were obtained during an epidemic of Japanese B encephalitis, possessed antibodies against the virus responsible for the epidemic. This observation suggests that horses may occupy the role of intermediary hosts for the virus. The importance of this role in the epidemiology of the disease in human beings depends to a large extent upon whether the virus is capable of circulating in the blood stream of infected horses, from which source it might be transmitted to man by mosquitoes. In order to obtain information concerning the matter, four Okinawan horses were inoculated with the virus of Japanese B encephalitis on 16 September, 1945. The present report is concerned with the results of the experiment.

Materials and Methods Through the cooperation of the Commander of Naval Construction Troops, a screened, wooden-frame

stable was built on the outskirts of the village of Ishikawa. This building contained four stalls, a separate room for laboratory studies, and a concrete floor with drainage outlets leading to a nearby pit. Throughout the course of the experiment, the interior was thoroughly sprayed each day with DDT.

Four horses were selected from the corral at Ishikawa. Like virtually all other horses on Okinawa, these were malnourished, undersized animals whose general physical condition was obviously poor. Three of the horses were approximately one year old; the fourth was a Mongolian pony approximately 12 years old. Each horse was inoculated intravenously with 2 cc of a 10% suspension of mouse-brain tissue, infected with a strain of Japanese B encephalitis virus isolated during this epidemic from a fatal case of encephalitis.² Specimens of blood were obtained from each horse before the injection of virus and at intervals of 16 hours, 3, 6, 9, and 12 days after inoculation. Neutralization tests were performed with the pre-inoculation sera in order to determine the status of immunity in each animal at the time of injection. The specimens of blood taken after inoculation were injected intracerebrally and intraperitoneally into mice, in order to determine the presence or absence of virus. In the instances where a virus was recovered

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² Hodess, H. L., Thomas, L., and Peck, J. L., *Proc. Soc. Exp. Biol. and Med.*, 1945, 60, 220.

TABLE I
Results of Neutralization Tests and Mouse Inoculations with the Blood of Horses Inoculated with Japanese B Encephalitis Virus

Horse No	Neutralizing titer*	Virus in blood				
		16 hr	3 days	6 days	9 days	12 days
1	0	0	+	+	0	—
2	1000	0	0	0	0	0
3	1000	0	0	0	0	0
4	1000	0	0	0	0	0

* Titer is expressed as number of 50% mortality doses of virus against which each serum protected mice

from the blood stream, it was subsequently identified as that of Japanese B encephalitis by means of neutralization tests in mice

Results The results of neutralization tests with sera obtained prior to inoculation, and the results of the injection of mice with blood taken following inoculation, are shown in Table I. It will be seen that Horse No 1, a yearling, did not possess demonstrable neutralizing antibodies in the serum obtained before inoculation. Sixteen hours after injection, the virus had apparently disappeared from the circulating blood, as shown by the negative results obtained by the injection into mice of blood removed at this time. However, 3 days following inoculation, the virus had reappeared in the blood, since each of 3 mice, which were injected with blood removed on the third day, developed typical symptoms of encephalitis and died 6 days after injection. The virus in the brains of these mice was identified as that of Japanese B encephalitis by means of neutralization tests with known hyperimmune Japanese B antiserum. On the 6th day after the inoculation of Horse No 1, virus was again detected in the blood of the animal and subsequently identified as the virus of Japanese B encephalitis. On the 9th day, virus was not demonstrated in the blood of Horse No 1. It is noteworthy that, on this day, its serum was proved to contain sufficient antibody to neutralize 100 50%-mortality doses of virus in mice. It is possible that the failure to demonstrate virus in the blood on this day may have been due to a masking of small amounts of virus by antibody.

Horse No 1 became ill on the sixth day, failing to eat and showing generalized weakness. The weakness increased during the next four days, and on the 10th day the animal

was moribund. At this time it was sacrificed, and specimens of tissues were obtained for inoculation of mice as well as for histopathological examination. Virus could not be recovered from the brain, spinal cord, or spleen of this horse, and microscopic examination of the brain showed no evidence of encephalitis. The cause of the death of this horse is not known, it is possible that it may have been due in part to prolonged malnutrition.

On the day of the experimental inoculation, Horses No 2, No 3, and No 4, as is shown in Table I, each possessed antibody in sufficient titer to neutralize 1000 50%-mortality doses of virus in mice. As might be expected, virus was not recovered from the blood of any of these animals at any time up to the 12th day after inoculation. These horses developed no symptoms of infection and remained entirely well for a period of 20 days after inoculation.

Summary It has been demonstrated that an Okinawan horse was susceptible to infection by the virus of Japanese B encephalitis and that the virus was present in the circulating blood 3 and 6 days after inoculation. It could not be demonstrated 16 hours after inoculation, nor after 9 days, at which time a small amount of neutralizing antibody had appeared. In 3 other horses which possessed high titers of neutralizing antibodies prior to the injection, virus could not be demonstrated in the blood stream following inoculation. These results, when considered in conjunction with the previously demonstrated presence of high titers of antibodies in the sera of many Okinawan horses,¹ constitute further evidence that this species of animal may play a role in the epidemiology of Japanese B encephalitis.

Relationship of Carotid and Aortic Mechanisms to Digitalis Emesis *

N W PINSCHMIDT (Introduced by H B Haag)

From the Department of Pharmacology Medical College of Virginia, Richmond

The site of the emetic action of digitalis and related compounds has been a subject of considerable research and controversy over a period of more than 30 years. The initial conclusions of Hatcher and Eggleston^{1,2,3} that the effect was of central rather than peripheral origin, were not borne out by later studies^{4,5}. Hatcher and Weiss^{6,7} and Hatcher and French⁸ suggested that the emetic response to digitalis was of a reflex nature initiated by afferent impulses arising in the heart. This was later questioned by Dresbach and Waddell,^{9,10,11} and by Haney and Lindgren.¹² The latter authors conclude that the mechanism must lie either in a direct action on the medullary center, as originally suggested or 'on other structures from which impulses pass to the center via nerves other than those elim-

inated in the experiments described." The liver, as a possible source of such impulses has been eliminated by Dresbach.¹³ Hanzlik and Wood concluded that in spite of the fact that digitalis emesis occurred in their de-hepatized pigeons the liver is still the primary source of the reflex.¹⁴ The gastro-intestinal tract was eliminated by Hatcher and Eggleston.¹

It is well known the muscles of respiration are definitely concerned with vomiting¹⁵ and that excitation of the mechanisms located in the carotid and aortic bodies and indirectly through the carotid sinus and arch of the aorta may, under proper circumstances, result in respiratory stimulation. It has also been noted⁹ that in cases where emesis does not result from digitalis administration *rapid respiration* frequently occurs. The present study was undertaken in order to determine whether or not the surgical elimination of the carotid and aortic mechanisms or their nervous pathways might result in abolishing the emetic response. An abstract of the work herein reported appeared recently.¹⁶

Method A single intravenous dose of digitoxin which would produce emesis within 30 minutes, was established. Three dogs were given 0.09 mg/kg digitoxin. At the end of one hour, none of the animals having vomited, each dog was given one-half of its original dosage. Emesis occurred in all animals within 30 minutes. Three dogs were, therefore, given the approximate sum of these doses (0.15 mg/kg). Emesis occurred in all animals within 30 minutes. Hence, in all subsequent experiments this was the vomiting dose employed.

* The author is indebted to Dr H B Haag for his valuable counsel during the course of this work and to Dr E L Smith who assisted with the surgical procedures.

¹ Hatcher, R A and Eggleston Carr, *J Pharmacol Exp Therap*, 1912, 4, 113.

² Eggleston Carr, and Hatcher R A, *J A M A*, 1913, 60, 499.

³ Eggleston Carr, *J A M A*, 1913, 61, 757.

⁴ Hatcher, R A, and Weiss S, *Arch Int Med* 1922, 29, 690.

⁵ Hatcher, R A, and Weiss, S, *J Pharmacol Exp Therap* 1923, 22, 193.

⁶ Hatcher R A, and Weiss, S, *J Pharmacol Exp Therap*, 1927, 32, 37.

⁷ Hatcher, R A, and Weiss, S, *J A M A*, 1927, 89, 429.

⁸ Hatcher, R A, and French, S, *J Pharmacol Exp Therap*, 1932, 46, 97.

⁹ Dresbach, M and Waddell, K. C., *J Pharmacol Exp Therap*, 1926, 27, 9.

¹⁰ Dresbach, M, and Waddell, K C., *J Pharmacol Exp Therap*, 1928, 31, 43.

¹¹ Dresbach, M, and Waddell, K. C., *J Lab and Clin Med*, 1929, 14, 625.

¹² Haney, H. F., and Lindgren A J., *J Pharmacol Exp Therap*, 1942, 76, 363.

¹³ Dresbach, M, *Proc Soc Exp Biol and Med*, 1936, 37, 35, 92.

¹⁴ Hanzlik, P J and Wood D A, *J Pharmacol Exp Therap*, 1929, 37, 67.

¹⁵ Hatcher, R A, *Physiol Rev*, 1934, 4, 479.

¹⁶ Pinschmidt, N W, *Fed Proc*, 1944, 3.

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3	1000	0	0	0	0	0
4	1000	0	0	0	0	0

* Titer is expressed as number of 50% mortality doses of virus against which each serum protected mice

from the blood stream, it was subsequently identified as that of Japanese B encephalitis by means of neutralization tests in mice

Results The results of neutralization tests with sera obtained prior to inoculation, and the results of the injection of mice with blood taken following inoculation, are shown in Table I. It will be seen that Horse No 1, a yearling, did not possess demonstrable neutralizing antibodies in the serum obtained before inoculation. Sixteen hours after injection, the virus had apparently disappeared from the circulating blood, as shown by the negative results obtained by the injection into mice of blood removed at this time. However, 3 days following inoculation, the virus had reappeared in the blood, since each of 3 mice, which were injected with blood removed on the third day, developed typical symptoms of encephalitis and died 6 days after injection. The virus in the brains of these mice was identified as that of Japanese B encephalitis by means of neutralization tests with known hyperimmune Japanese B antiserum. On the 6th day after the inoculation of Horse No 1, virus was again detected in the blood of the animal and subsequently identified as the virus of Japanese B encephalitis. On the 9th day, virus was not demonstrated in the blood of Horse No 1. It is noteworthy that, on this day, its serum was proved to contain sufficient antibody to neutralize 100 50%-mortality doses of virus in mice. It is possible that the failure to demonstrate virus in the blood on this day may have been due to a masking of small amounts of virus by antibody.

Horse No 1 became ill on the sixth day, failing to eat and showing generalized weakness. The weakness increased during the next four days, and on the 10th day the animal

was moribund. At this time it was sacrificed, and specimens of tissues were obtained for inoculation of mice as well as for histopathological examination. Virus could not be recovered from the brain, spinal cord, or spleen of this horse, and microscopic examination of the brain showed no evidence of encephalitis. The cause of the death of this horse is not known, it is possible that it may have been due in part to prolonged malnutrition.

On the day of the experimental inoculation, Horses No 2, No 3, and No 4, as is shown in Table I, each possessed antibody in sufficient titer to neutralize 1000 50%-mortality doses of virus in mice. As might be expected, virus was not recovered from the blood of any of these animals at any time up to the 12th day after inoculation. These horses developed no symptoms of infection and remained entirely well for a period of 20 days after inoculation.

Summary It has been demonstrated that an Okinawan horse was susceptible to infection by the virus of Japanese B encephalitis and that the virus was present in the circulating blood 3 and 6 days after inoculation. It could not be demonstrated 16 hours after inoculation, nor after 9 days, at which time a small amount of neutralizing antibody had appeared. In 3 other horses which possessed high titers of neutralizing antibodies prior to the injection, virus could not be demonstrated in the blood stream following inoculation. These results, when considered in conjunction with the previously demonstrated presence of high titers of antibodies in the sera of many Okinawan horses,¹ constitute further evidence that this species of animal may play a role in the epidemiology of Japanese B encephalitis.

Relationship of Carotid and Aortic Mechanisms to Digitalis Emesis *

N W PINSCHMIDT (Introduced by H B Haag)

From the Department of Pharmacology, Medical College of Virginia, Richmond

The site of the emetic action of digitalis and related compounds has been a subject of considerable research and controversy over a period of more than 30 years. The initial conclusions of Hatcher and Eggleston,^{1,2,3} that the effect was of central rather than peripheral origin, were not borne out by later studies.^{4,5} Hatcher and Weiss^{6,7} and Hatcher and French⁸ suggested that the emetic response to digitalis was of a reflex nature initiated by afferent impulses arising in the heart. This was later questioned by Dresbach and Waddell,^{9,10,11} and by Haney and Lindgren.¹² The latter authors conclude that the mechanism must lie either in a direct action on the medullary center, as originally suggested, or "on other structures from which impulses pass to the center via nerves other than those elim-

inated in the experiments described." The liver, as a possible source of such impulses, has been eliminated by Dresbach.¹³ Hanzlik and Wood concluded that, in spite of the fact that digitalis emesis occurred in their de-hepatized pigeons, the liver is still the primary source of the reflex.¹⁴ The gastro-intestinal tract was eliminated by Hatcher and Eggleston.¹

It is well known the muscles of respiration are definitely concerned with vomiting¹⁵ and that excitation of the mechanisms located in the carotid and aortic bodies and indirectly through the carotid sinus and arch of the aorta may, under proper circumstances, result in respiratory stimulation. It has also been noted⁹ that in cases where emesis does not result from digitalis administration *rapid respiration* frequently occurs. The present study was undertaken in order to determine whether or not the surgical elimination of the carotid and aortic mechanisms or their nervous pathways might result in abolishing the emetic response. An abstract of the work herein reported appeared recently.¹⁶

Method A single intravenous dose of digitoxin, which would produce emesis within 30 minutes, was established. Three dogs were given 0.09 mg/kg digitoxin. At the end of one hour, none of the animals having vomited, each dog was given one-half of its original dosage. Emesis occurred in all animals within 30 minutes. Three dogs were, therefore, given the approximate sum of these doses (0.15 mg/kg). Emesis occurred in all animals within 30 minutes. Hence, in all subsequent experiments this was the vomiting dose employed.

* The author is indebted to Dr H B Haag for his valuable counsel during the course of this work and to Dr E L Smith who assisted with the surgical procedures.

¹ Hatcher, R A, and Eggleston, Cary, *J Pharmacol Exp Therap*, 1912, 4, 113

² Eggleston, Cary, and Hatcher, R A, *J A M A*, 1913, 60, 499

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⁴ Hatcher, R A, and Weiss, S, *Arch Int Med*, 1922, 29, 690

⁵ Hatcher, R A, and Weiss, S, *J Pharmacol Exp Therap*, 1923, 22, 193

⁶ Hatcher, R A, and Weiss, S, *J Pharmacol Exp Therap*, 1927, 32, 37

⁷ Hatcher, R A, and Weiss, S, *J A M A*, 1927, 89, 429

⁸ Hatcher, R A, and French, S, *J Pharmacol Exp Therap*, 1932, 46, 97

⁹ Dresbach, M, and Waddell, K C, *J Pharmacol Exp Therap*, 1926, 27, 9

¹⁰ Dresbach, M, and Waddell, K C, *J Pharmacol Exp Therap*, 1928, 31, 43

¹¹ Dresbach, M, and Waddell, K C, *J Lab and Clin Med*, 1929, 14, 625

¹² Haney, H F, and Lindgren, A J, *J Pharmacol Exp Therap*, 1942, 76, 363

¹³ Dresbach, M, *Proc Soc Exp Biol and Med*, 1936 37, 35, 92

¹⁴ Hanzlik, P J, and Wood, D A, *J Pharmacol Exp Therap*, 1929, 37, 67

¹⁵ Hatcher, R A, *Physiol Lett*, 1924, 4, 479

¹⁶ Pinschmidt, N W, *Fed Proc*, 1944, 3

TABLE I
Effect of Removal of Carotid Bodies and Sinuses and Denervation of the Aortic Regulators on the Emetic Response to 0.15 mg/kg Digitoxin on Dogs

Dog No	Date	Treatment	Emesis after digitoxin in
1 ♂	4 12		22 minutes
	20	Carotids removed	24 "
	26		
2 ♀	5 24		18 "
	29	Carotids removed	6 "
	6 3		
	9	Right vagus cut	17 "
3 ♀	12		
	5 29		20 "
	6 5	Carotids removed	16 "
	12		
4 ♀	19	Right vagus cut	15 "
	23		
	12 18		16 "
	21	Carotids removed left vagus cut	25 "
5 ♂	27		
	1 10	Right vagus cut	25 "
	11		
	12 27		12 "
6 ♀	28	Carotids removed, left vagus cut	12 "
	1 3		
	12 29		6 "
	30	Carotids removed, left vagus cut	22 "
7 ♂	1 13		
	12 30		15 "
	31	Carotids removed, left vagus cut	27 "
	1 6		
	11	Right vagus cut	16 "
	13		

Seven dogs (4 females, 3 males) were each checked for the normal emetic response to 0.15 mg/kg digitoxin. After allowing a minimum of 24 hours for recovery, the animals underwent the following operations in 2 or more steps as indicated by Table I: (a) removal of the carotid bodies and sinuses by extirpation of segments of the carotid arteries from points just caudal to the origin of the superior thyroid artery to points slightly cephalic to the origin of the lingual artery, (b) sectioning of one or both vagal trunks (in the neck region) thus interrupting the nervous pathways from the aorta.¹⁷ All operations were performed under ether anesthesia.

Each animal was given a minimum of 24 hours to recover from operation and at least

7 days to recover from the previous dose of digitoxin. The dog was then given 0.15 mg/kg digitoxin and watched for 30 minutes for emesis. This was repeated after each phase of the operation.

The results are shown in Table I. In every case vomiting was as prompt and typical after operation as before. Six of these dogs survived sectioning of one vagus and 2 survived sectioning of both vagi without change in their vomiting response to digitoxin. This is contrary to the experience of Hanzlik and Wood¹⁴ who found that double vagotomy abolished digitalis emesis in pigeons. It is in agreement, however, with the results of Haney and Lindgren¹³ on dogs.

Conclusion Vomiting initiated by digitoxin is not abolished by removal of the carotid bodies and sinuses and by denervation of the

¹⁷ Comroe, J. H., Jr., *Am J Physiol*, 1939, 127, 176

aortic mechanisms Assuming the mechanism to be reflex in nature, the site of origin of the impulses causing digitalis vomiting is still undetermined The possibility remains that afferent impulses may be set up in a structure

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15209

The Electron Micrography of Plant Virus-Antibody Mixtures

L M BLACK,* W C PRICE,[†] AND RALPH W G WICKOFF[‡]

From the National Institute of Health, Bethesda, Md, and the Department of Animal and Plant Pathology, the Rockefeller Institute for Medical Research, Princeton, N J

Electron microscopy is a promising new way of studying the interaction between an antigen and its specific antibody Such a study is of practical importance because the minute amounts of required material make possible the development of unusually sensitive tests for antigen and antibody From a more fundamental standpoint the ability of the microscope to record particles comparable in size to antibody molecules may provide a way of checking directly the several hypotheses that have been developed to explain the nature and specificity of the immune reaction

Very few papers dealing with the electron microscopy of antigen-antibody mixtures have thus far been published In two^{1,2} the antigens are bacteria, another³ describes the apparent swelling of tobacco mosaic virus particles when mixed with antibody The published pictures of sensitized typhoid and paratyphoid bacilli give clear evidence of the adsorption of antibody onto both the cell walls and the flagella

In the tobacco mosaic-serum photographs isolated fibers show the same kind of thickening as these bacterial flagella It was suggested³ this broadening of the tobacco mosaic particles, from a normal 150A to *ca* 600A, was due to each being coated with a single layer of antibody molecules arranged with their long axes at right angles to the axis of the virus particle

The visibility of viruses⁴ and other objects of macromolecular dimensions⁵ is improved by the oblique evaporation upon the preparation of a very thin metallic film⁶ We are using this "shadow" technic to see what new information it will give concerning the reaction between certain plant viruses and sera prepared against them Preliminary photographs are reproduced in this note

Antigens used were tomato bushy stunt and southern bean mosaic viruses purified by means of a preliminary clarification with $(\text{NH}_4)_2\text{SO}_4$ followed by fractionation in a high speed centrifuge Antisera for the bushy stunt virus and for the bean virus⁷ were prepared by intraperitoneal injection of rabbits with puri-

* Department of Animal and Plant Pathology, The Rockefeller Institute for Medical Research, Princeton, New Jersey

[†] Department of Biology, University of Pittsburgh, Pittsburgh, Pa

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¹ Mudd, S, Hemmets, F, and Anderson, T F, *J Exp Med*, 1943, **78**, 327

² Mudd, S, and Anderson, T F, *J Immunology*, 1941, **42**, 251

³ Anderson, T F, and Stanley, W M, *J Biol Chem* 1941, **139**, 339

⁴ Williams, R C, and Wickoff, R W G, *Proc Soc Exp Biol and Med*, 1945, **58**, 265, *Science*, 1945, **101** 594, Price, W C, Williams, R C and Wickoff, R W G, *Science*, 1945, **102** 277

⁵ Williams, R C and Wickoff, R W G, *Nature* 1945, **156**, 68

⁶ Williams, R C and Wickoff, R W G, *J Applied Physics*, 1944, **15**, 712, 1946, **17** No 1

⁷ Price, W C, and Black, L M, *Phytopathology*, in press

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	6-5		
	12	Right vagus cut	16 "
	19		
4 ♀	23		15 "
	12-18	Carotids removed left vagus cut	16 "
	21		
	27		25
	1-10	Right vagus cut	
	11		25 "
5 ♂	12-27	Carotids removed left vagus cut	12 "
	28		
	1-3		12 "
6 ♀	12-29	Carotids removed left vagus cut	6 "
	30		
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⁴ Williams, R C, and Wyckoff, R W G, *Proc Soc Exp Biol and Med*, 1945, **58**, 265, *Science*, 1945, **101**, 594, Price, W C, Williams, R C, and Wyckoff, R W G, *Science*, 1945, **102**, 277

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⁷ Price, W C, and Black, L M, *Phytopathology*, in press



Fig 1

An electron micrograph of a field in a preparation of purified southern bean mosaic virus. At the top of the picture the bean virus particles, singly and in groups, are widely separated from one another on the substrate. In the middle they are more closely packed into a newly continuous layer. At the bottom these layers of particles are streaked on top of one another to give the beginnings of a crystalline array. Magnification 10,800 X

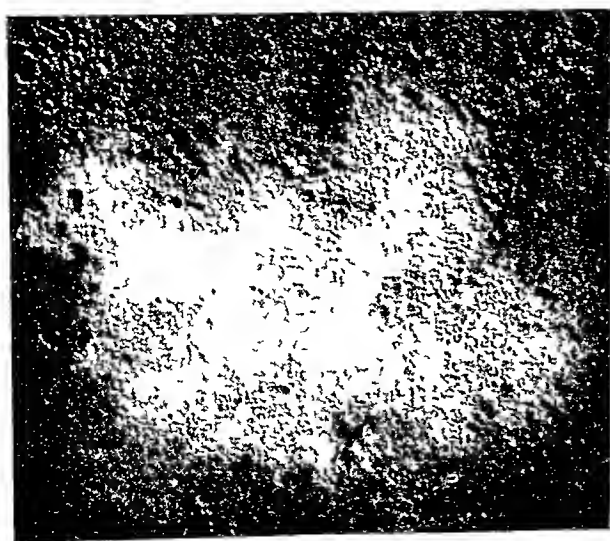


Fig 2

An electron micrograph of a mixture of purified southern bean mosaic virus with its antiserum. The individual particles in the field do not have the same degree of regularity in arrangement that is evident in Fig 1. Magnification 16,800 X

fied virus suspensions. The anti-bushy stunt serum was originally made for another set of experiments for which it was important that the serum be freed from traces of tobacco mosaic antiserum that might be present. To insure this it has been absorbed with purified tobacco mosaic virus, the excess of which provides the rod-forms seen in Fig 3.

Preparations for electron microscopy were made in the following way. Purified virus containing *ca* one milligram per cc was mixed

with an equal volume of undiluted serum and the mixture immediately diluted with from 10 to 100 volumes of distilled water. This diluted serum-virus mixture was examined immediately and after varying intervals up to 24 hours. Time seemed to have little effect on the character of the electron photograph obtained. The concentrated serum-virus mixture soon became turbid with the eventual precipitation of a part of its contents but the more dilute mixtures remained clear through-

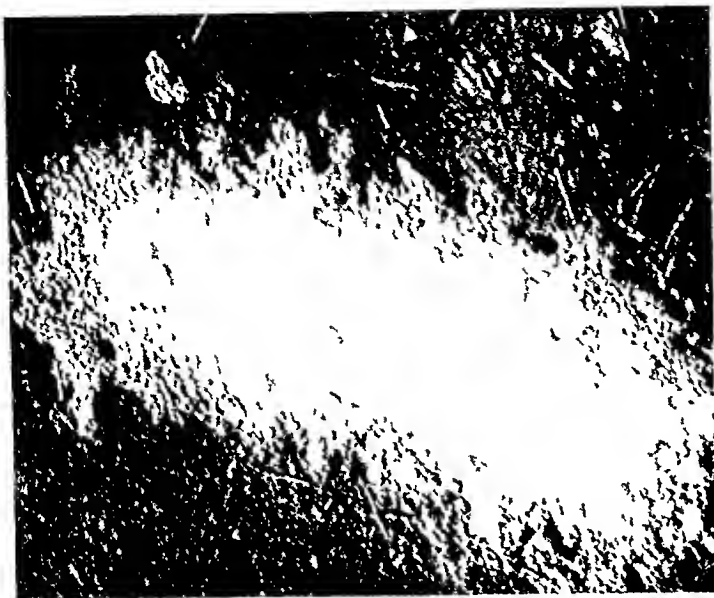


FIG. 3
An electron micrograph of a mixture preparation consisting of a mixture of purified bushy stunt virus with antiserum. Magnification 20,500 X.

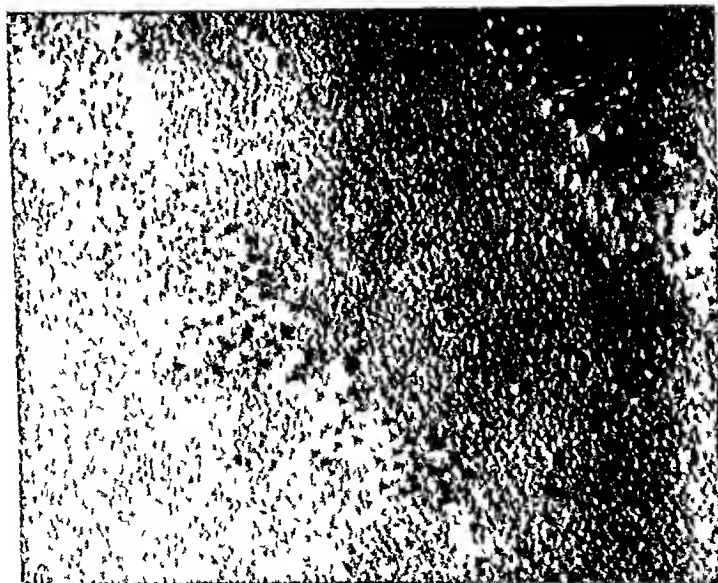


FIG. 4
An electron micrograph of a field of a preparation consisting of a mixture of bushy stunt virus with normal rabbit serum. Magnification 20,500 X.

out the period of observation. Micro-drops of the diluted mixtures were placed on the usual collodion-covered screens, left several minutes and then withdrawn as completely as possible. After being allowed to dry in the air the screens containing the less dilute mixtures were washed several times by the addition and subsequent withdrawal of droplets

of distilled water. Final preparations were shadowed as usual in the vacuum chamber of a metal-evaporating unit[§]. Gold in an aver-

[§] We wish to express our gratitude to Perry C. Smith and R. G. Picard of the Radio Corporation of America, Camden, N. J. for the use of evaporating equipment.

age thickness of *ca* 8A was laid down, the angle of evaporation being such that an object on the preparation would cast a shadow five times its height

Two sorts of preparations were made and examined as controls for the foregoing virus-antibody mixtures. One consisted of mixtures containing normal in place of immune serum, the other was of serum without admixed virus. These were shadowed in the usual fashion before examination.

Both viruses have elementary bodies that are essentially spherical, in pure suspension and at sufficient concentrations they show a pronounced tendency to associate in regular crystal-like arrays⁸. When mixed with specific antiserum, however, these particles appear aggregated without the same regularity into microclumps. The character of this micro-agglutination will be clear from a comparison of Fig 1 and 2. Fig 1 is a typical field from a purified preparation of the southern bean mosaic virus, Fig 2 shows a mixture of this virus with its antibody. Similar microflocs are produced when the bushy stunt virus and its antiserum are mixed (Fig 3). Micro-flocculation has also been observed with the tobacco mosaic virus protein and with other antigens when mixed with their anti-substances. Similar agglutinations were not seen when normal rabbit serum replaced specific serum in the mixtures. For example, in Fig 4, which is taken of a mixture of bushy stunt virus and normal serum, virus particles are separate from one another

in their enmeshing serum. The amount of virus in single microflocs on preparations such as those giving Fig 2 and 3 often is no more than *ca* 10^{-12} g.

In these photographs the background structure due to the collodion is often less sharply defined than usual. This apparent smoothing-over of detail in the substrate is probably due to an irreversible deposition of serum proteins over the entire surface of the preparation.

It is not so simple as might at first be imagined to measure accurately the apparent increase in particle size of the virus in these mixtures. The agglutinated particles of Fig 2 and 3 are close to one another but their outlines are often diffuse and the irregularity of their vertical stacking prevents many determinations of particle-separation. Best results are obtained by seeking places where several particles are lined up in rows. Preliminary measurements on such rows indicate that the apparent separation of both agglutinated bean mosaic and bushy stunt virus particles is about twice that found in pure suspensions. More accurate studies are being made of mixtures containing different proportions of antigen in order to obtain more precise data.

Summary Shadowed electron micrographs of mixtures of purified southern bean mosaic and bushy stunt viruses with their specific antisera show their spherical elementary bodies aggregated into microflocs in which the particle-separations seem to be about twice their normal values. More detailed electron micrographic study of these systems is underway.

⁸ Price, W. C., Williams, R. C., and Wyckoff, R. W. G., *op cit*, *Arch. Biochem.*, in press.

The Effect of Lecithin Administered Intravenously

GERALD K. ASHBY (Introduced by Warren M. Cox, Jr.)

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It has been reported that various lecithins when administered by vein cause hemolysis,^{1,2,3} leukocytosis,⁴ changes in the fragility of the blood cellular components,^{4,5,6} or death.⁷ On the other hand, one investigator reports that regardless of the method of administration lecithin alone exerts no toxic action.⁸ Because of the current interest in fat emulsions for parenteral use,^{9,10,11} a brief study of the effect of lecithin when administered by vein has been made.

Experimental Each sample of lecithin was prepared as a 70% colloidal solution in pyrogen-free distilled water and autoclaved at 10 pounds pressure for 15 minutes. The sterile solutions were rapidly injected into the marginal ear veins of normal rabbits in amounts sufficient to supply 1.75 g of lecithin per kilogram body weight. Table I shows the essential data obtained with 13 samples of lecithin. All but one of the injected rabbits died within 12 hours. These results strongly suggest that none of the tested lecithins is suitable for making fat emulsions for intravenous use.

Various methods of isolating lecithin from eggs were investigated, but the methods generally used for this purpose were not entirely successful in our hands.^{12,13} The method finally adopted consisted of exhaustive extraction of powdered egg yolk with acetone to remove the fat, subsequent extraction with alcohol to obtain the crude lecithin, distillation of the alcohol under high vacuum, solution of the residue in ether and final precipitation of the lecithin with acetone. The precipitated lecithin was redissolved in ether, filtered and reprecipitated with acetone several times. Final traces of solvent were removed under high vacuum at low temperature. Yields by this method were 11% of the original weight of the egg powder.

Lecithin prepared in this manner and stored under high vacuum for varying lengths of time up to 228 days was injected into 14 normal rabbits without any apparent ill effects (Table II). The level of 1.75 g per kilo body weight was increased to 3.5 g per kilo and again without any ill effects, but 7.0 g proved to be fatal.

Samples of egg lecithin prepared and tested as above were then exposed to the air at room temperature and at 55°C for varying periods of time to determine if toxic properties would develop. As indicated in Table II, toxic properties did develop in as little as 14 days at 55°C and 47 days at room temperature. The toxicity which developed in 53 days at 55°C was profound, since both rabbits died in exactly 4 minutes after the infusions were begun. It was thought that perhaps this toxicity might be due to lysolecithin but efforts to purify the exposed sample from toxic substances by the differential solubility of lecithin

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³ Levin, B. S., *C. R. Soc. Biol.*, 1935, **119**, 80.

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¹⁰ McKibbin, J. M., Pope, A., Thayer, S., Ferry, R. M., Jr., and Stare, F. J., *J. Lab. and Clin. Med.*, 1945, **30**, 488.

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TABLE I
Lecithins from Various Sources

Type of lecithin	Source*	Label description	Color	Consistency	Color of solution	No rabbits injected	Lecithin g/kg/BW	Results
Egg	1	about 90%	dk red	soft wax	dk cream	2	1.75	Died in 4 and 105 minutes
"	2	—	" "	" "	" "	1	1.75	Died during night
"	3	pure	red bk	" "	brown	1	1.75	Died in 22 minutes
"	4	practical	brown	" "	" "	1	1.75	Died in 30 minutes
Soy bean	5	pure	dk brown	" "	" "	1	1.22	Died during injection
"	6	fat free phosphatides	white	granular	white	5	1.75	Died within 8 hrs†
"	6	commercial	amber	only wax	" "	1	1.75	Died during night
"	6	edible	lt amber	heavy oil	" "	1	1.75	" " "
"	6	" "	dk amber	" "	cream	1	1.75	Died in 4 hours
Animal	5	90%	brown	wax	brown	1	1.75	Died during night
Vegetable	5	60% or more	lt amber	heavy oil	white	1	1.75	" " "
"	5	60% " "	dk amber	" "	cream	1	1.75	" " "
"	5	app 75%	lt amber	hard wax	white	1	1.75	Complete paralysis recovered in 1 wk

* (1) Merck & Co., kindly supplied by Dr L. Emmett Holt, Jr., (2) Difco Laboratories, Inc., (3) Coleman and Bell Company, (4) Eastman Kodak Co., (5) Pfaffstiel Chemical Company, (6) American Lecithin Company, kindly supplied for research by Dr A. Schuf

† One died in 7 min

TABLE II
Experimental Lecithin Preparations

Method of preparation or treatment	Color of lecithin	Color of solution	No rabbits injected	Lecithin g/kg/BW	Results
Acetone ppt'd from ether solution	amber	white	14*	1.75	No apparent ill effects
" " " " " "	" "	cream	35†	" "	" " "
" " " " " "	" "	" "	1	7.0†	Died during night
Exposed to air at room temp 47 days	dk red	brown	2	1.75	One died during the night, one no apparent ill effects
" " " " " " 86 "	brown	" "	2	1.75	" " " " " " " " " "
" " " " " " 55°C 14 days	dk red	" "	2	1.75	One no apparent ill effects, one died during week
" " " " " " 53 "	" "	" "	" "	" "	end, 72-120 hours
Cadmium chloride pptn 12	black	dk brown	2	0.625	Both died 4 min after start of inj
Cadmium chloride pptn ‡	amber	white	1	1.75	Died during night
" " " " " "	" "	" "	1	1.75	No apparent ill effects

* Several of these rabbits were injected 2 or more times

† Cadmium removed by pptn with H_2S

‡ In 14% solution

and lysolecithin in ether¹⁴ were unsuccessful

Chemically speaking, the purest lecithin has been prepared through the cadmium salt.¹² This procedure did give a very light-colored and attractive preparation, but a fat emulsion prepared with it was promptly fatal to a dog and the lecithin itself was fatal to a rabbit (Table II). The toxicity of lecithin prepared in this manner was found to be due to traces of cadmium which could be removed by treatment with hydrogen sulphide.

Summary Thirteen samples of commercial lecithin were fatal to rabbits when adminis-

tered intravenously at a level of 1.75 g per kilo body weight.

A method for preparing lecithin from egg yolk is described and it is shown that lecithin so prepared is harmless to rabbits when given by vein even at a level of 3.5 g per kilo body weight. Lecithin so prepared will acquire toxic properties if exposed to the air. Elevated temperatures accelerate this process. If the lecithin is stored under high vacuum, toxic properties will not develop within at least 228 days.

Egg lecithin prepared through the cadmium salt may contain sufficient quantities of cadmium to produce cadmium poisoning.

¹⁴ Fiori, A., *Biochim. terap. exper.*, 1930, 17, 267.

15211

In vitro Susceptibility of *Histoplasma capsulatum* to Therapeutic Agents

JOHN H. SEABURY AND DOROTHY ARTIS (Introduced by H. A. Davis)

From the Department of Medicine, University of Michigan, and the Division of Hospital Laboratories, University Hospital, Ann Arbor, Mich.

During treatment of 3 cases of histoplasmosis in the early months of 1945, it was decided to carry out *in vitro* susceptibility studies on the yeast phase of *Histoplasma capsulatum*.

Methods Five-day-old cultures of *Histoplasma capsulatum* in heart-brain infusion broth (Difco) were used as the test organism.

The drugs tested were neoarsphenamine, neostam, sulfadiazine, sulfathiazol, stilbamidine*. Two antibiotics, streptomycin* and penicillin were likewise tested. The drugs were added to the media, before inoculation of the organism, in the following mg% concentrations: 100, 50, 25, 20, 10, 1, 0.1, 0.01, and 0.001.

The streptomycin was employed in the same manner, but in concentrations of 2500, 1250, 750, 250, 125, 100, 75, 50, 25, 12.5, 10, 7.5, and 5 units per ml. Penicillin was used in concentrations of 10, 5, and 1 units per ml.

Culture tubes of heart-brain infusion at pH

7.4, containing the desired concentrations of drugs in 10 ml, were inoculated, in duplicate with 0.5 ml of a 5-day broth culture of the yeast phase of the test organism and incubated at 37°C. These cultures were examined for growth at the end of 7 days of incubation. Similar tests were performed in which the amount of inoculum was reduced to 0.2 ml of the 5-day culture of *Histoplasma capsulatum*. Each of these series, in duplicate, was repeated once with identical results.

A control series, in which drug-free culture medium was inoculated with the same quantity of inoculum as used in the drug series was performed with each experiment.

The cultures were examined at the end of 7 days, and subcultures were made from all tubes which did not show growth or which were very cloudy. In tests using neoarsphenamine, stilbamidine, and streptomycin, some precipitate was formed when these agents were added to the heart-brain infusion broth. In the case of streptomycin, this was believed to be due to lipid substances present in the streptomycin. In the case of stilbamidine,

* The stilbamidine and streptomycin used were kindly supplied by Merck and Co.

TABLE I
Lecithins from Various Sources

Type of lecithin	Source*	Label description	Color	Consistency	Color of solution	No rabbits injected	Lecithin g/kg/BW	Results
Legg	1	about 90%	dk red	soft waxy	dk cream	2	1.75	Died in 4 and 105 minutes
"	2	—	" "	" "	" "	1	1.75	Died during night
"	3	pure	red bk	" "	brown	1	1.75	Died in 22 minutes
"	4	practised	brown	" "	" "	1	1.75	Died in 30 minutes
Soybean	5	pure	dk brown	" "	" "	1	1.22	Died during injection
"	6	fat free phosphatides	white	granular	white	5	1.75	Died within 8 hr†
"	6	commercial	amber	only waxy	" "	1	1.75	Died during night
"	6	edible	lt amber	heavy oil	" "	1	1.75	" "
Animal	5	90%	dk amber	" "	cream	1	1.75	Died in 4 hours
Vegetable	5	60% or more	brown	waxy	brown	1	1.75	Died during night
"	5	60%	lt amber	heavy oil	white	1	1.75	" "
"	5	app 75%	dk amber	" "	cream	1	1.75	" "
"	5	—	lt amber	hard waxy	white	1	1.75	" "
* (1) Merck & Co, kindly supplied by Dr L Emmott Holt, Jr, (2) Difco Laboratories, Inc, (3) Colman and Bell Company, (4) Eistman Kodak Co, (5) Pfaustrahl Chemical Company, (6) American Lecithin Company, kindly supplied for research by Dr A Schurff								
† One died in 7 min								
Complete paralysis recovered in 1 wk								

TABLE II
Experimental Lecithin Preparations

Method of preparation or treatment	Color of lecithin	Color of solution	No rabbits injected	Lecithin g/kg/BW	Results
Acetone ppt'd from ether solution	amber	white	14*	1.75	No apparent ill effects
" " " " " "	"	cream	1	3.5†	" " " "
Exposed to room temp 47 days	dk red	" "	1	7.0†	Died during night
" " " " " " 86 "	brown	brown	2	1.75	One died during the night, one no apparent ill effects
" " " " " " 14 days	dk red	" "	2	1.75	" " " "
" " " " " " 53 "	black	dk brown	2	1.75	One no apparent ill effects, one died during week
Cadmium chloride pptn ‡	amber	white	2	0.625	Both died 4 min after start of inj
Cadmium chloride pptn ‡	"	"	1	1.75	Died during night
"	"	"	1	1.75	No apparent ill effects

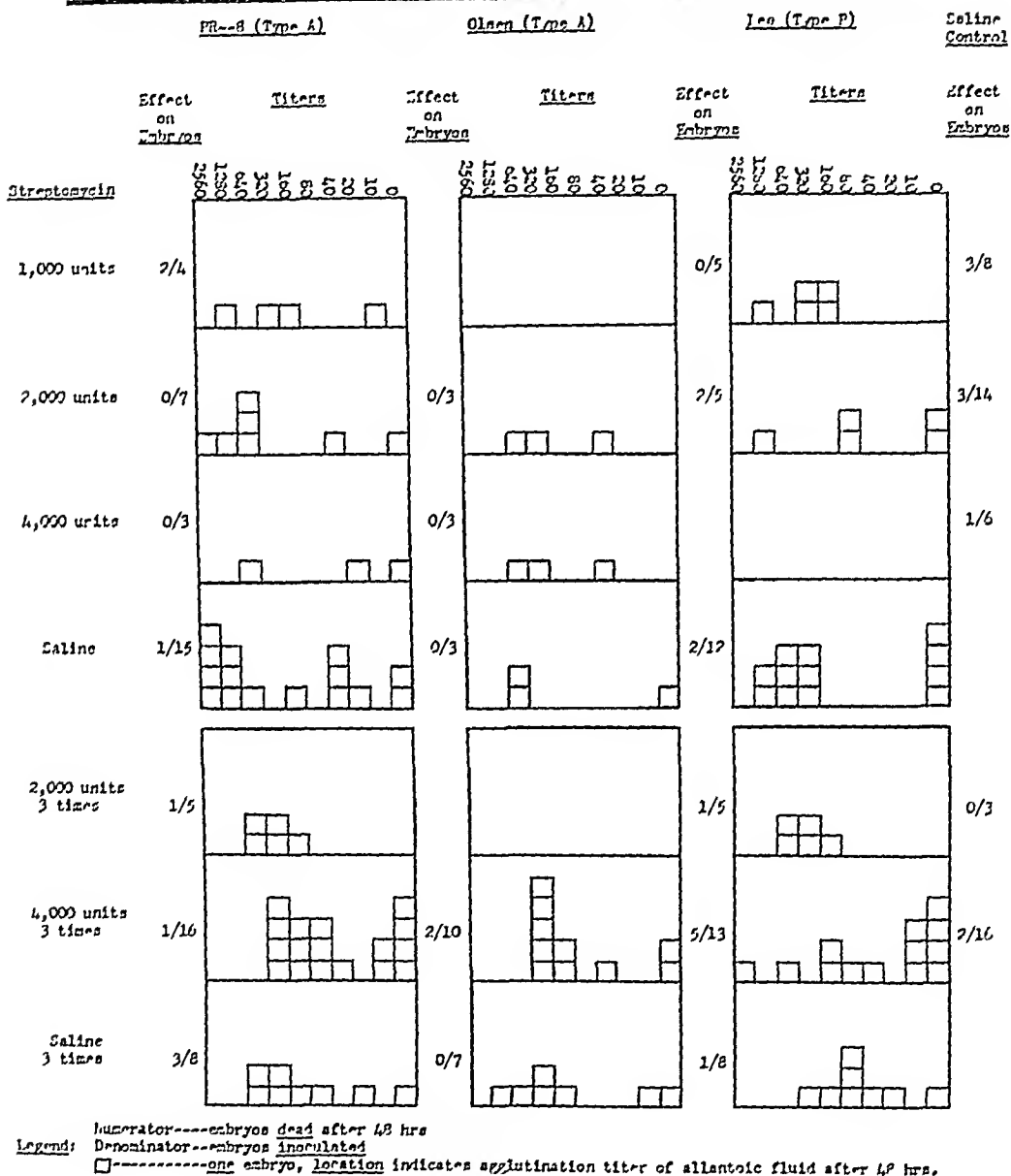
* Several of these rabbits were injected 2 or more times

† Cadmium removed by pptn with H₂S

‡ In 14% solution

CHART I

Inability of Streptomycin to Prevent Infection of Embryonated Chick Eggs with Influenza Strains



chick embryo, a laboratory trial against viruses which would grow in the chick embryo seemed warranted.

Method of Study The allantoic cavities of

¹ Jones, D, Metzger, I J, Schatz, A, and Wiseman, S A, *Science*, 1944, 100, 193

11- to 13-day-old embryonated chicken eggs were inoculated with various strains of influenza combined with varying amounts of streptomycin. The PR-8 and Olsen strains were used as representative of Type A and the Lee strain as an example of Type B.

TABLE I
In Vitro Effect of Various Concentrations of Therapeutic Agents on *Histoplasma capsulatum*

Concn mg%	100	50	25	20	10	1	0.1	0.01	0.001	*Control				
Neoarsphenamine	0	+	+	+	+	+	+	+	+	+				
Neostam	+	+	+	+	+	+	+	+	+	+				
Sulfadiazine	+	+	+	+	+	+	+	+	+	+				
Sulfathiazole	+	+	+	+	+	+	+	+	+	+				
Stilbamidine	0	0	0	0	0	+	+	+	+	+				
Concn units/ml	2500	1250	750	250	125	100	75	50	25	12.5	10	7.5	5	1
Streptomycin	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Penicillin											+		+	+

0 No growth

+

* Control tube contained no drug and was always positive

this was due to the sodium chloride present in the infusion broth, inasmuch as it is known that sodium chloride will produce crystallization of stilbamidine. The cause of the precipitate when neoarsphenamine was used is not known, but is probably related to the colloid-like properties of neoarsphenamine. This factor of precipitation undoubtedly reduced the amount of effective therapeutic agent present. At the time, it was impossible to determine the amount of each agent actually in solution.

Results The results of these experiments

are presented in Table I. On the basis of these experiments, it was found that neoarsphenamine in a concentration of 100 mg%, and stilbamidine in concentrations from 10 to 100 mg%, prevented the growth of *Histoplasma capsulatum*. As previously stated, the actual amount of neoarsphenamine and stilbamidine in solution was less than the tabulated concentrations.

The use of sulfadiazine, sulfathiazole, and neostam as well as streptomycin and penicillin exerted no inhibitory effect in the amounts used.

15212

Effect of Large Doses of Streptomycin and Influenza Viruses on Chick Embryos

ALFRED L. FLORMAN, ALICE B. WEISS, AND FRANCIS E. COUNCIL
(Introduced by V. E. Levine)

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Search for an effective therapeutic agent against viral infections has been stimulated by the success attained in treating bacterial diseases with sulfonamides and antibiotics. However, since the problem is complicated by the intracellular growth of viruses, it is perhaps not remarkable that the results up to the present time have been almost consistently negative.¹ Recently a small quantity of strep-

tomycin² became available to us. Because of the effectiveness of this agent against a number of bacteria which have been resistant to previously studied antibiotics and chemotherapeutic drugs, and its low toxicity for the

² Schatz, A., Bugie, L., and Waksman, S. A., *Proc. Soc. Exp. Biol. and Med.*, 1944, **55**, 66.

* The streptomycin was supplied by Merck and Company through the courtesy of Dr. D. F. Robertson. It consisted of remnants of lots No. 199, 200, and 212.

¹ Jones, D., Beaudette, F. R., Geiger, W. B., and Waksman, S. A., *Science*, 1945, **101**, 665.

TABLE I

Effect of Penicillin on the Lethal Action of Gonococcal Endotoxin in Mice

Gonococcal endotoxin	Dose, ml	Control mice Died/injected	Penicillin treated mice Died/injected
From strain 362	0.4	11/12	6/12
	0.1	8/12	2/12
From strain 354	0.5	10/10	6/10
	0.25	8/10	2/10
	0.1	7/10	0/10

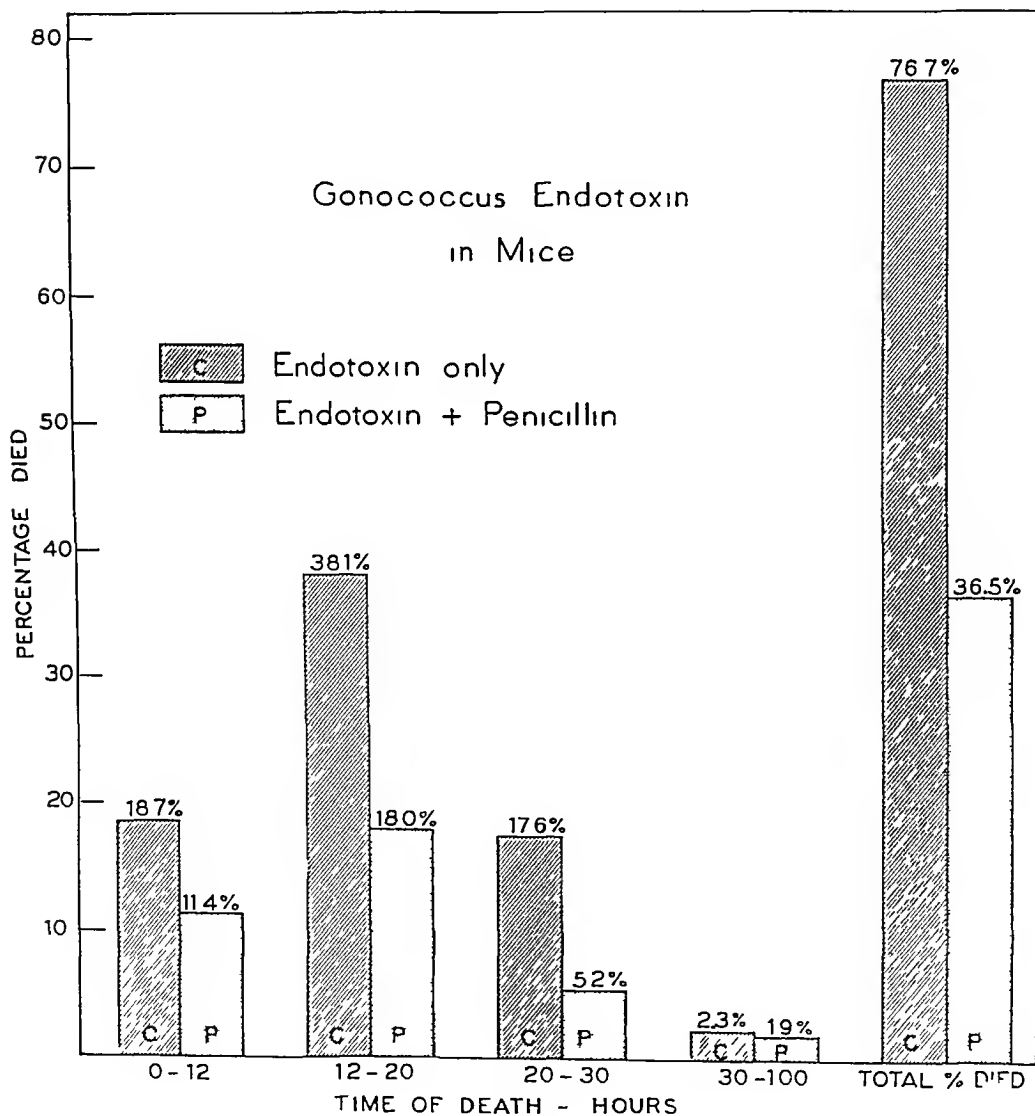


FIG 1

influenza[†] Each embryo received an original total inoculum of 0.15 cc, of this 0.05 cc was a 1:1000 dilution of infected allantoic fluid, and 0.10 cc was either saline or the antibiotic reconstituted and diluted with sufficient saline to give the desired concentration. The virus and streptomycin were mixed immediately before inoculation. In those instances where more than one "dose" of streptomycin was given, the subsequent injections were made in 0.15 cc quantities within the first 24 hours. In the interim, the shellholes were sealed with a small tent of Scotch tape. The eggs were incubated at 36° to 37°C for 48 hours. After this period the allantoic fluids were collected and their hemagglutinating titers for chicken red cells were determined.[‡]

[†] The influenza strains were furnished by Dr M. D. Eaton.

[‡] Florman, A. L., and Crawford, J. P., *Am J Med Sci*, 1944, **208**, 494.

Results of Study The results of several experiments are summarized in Chart I.[‡] From this it is apparent that

1. A total of 12,000 units given over a period of 24 hours failed to make any significant difference in the multiplication in the chick embryo of the PR-8, Olsen and Lee strains of influenza. This represents a total dosage of approximately 200,000 units per kilo of egg weight per 24 hours. It may be contrasted with the 150 and 300 units of streptomycin which protect chick embryos against *Shigella Gallinarum* and *Brucella abortus* infections.³

2. Despite the very large amounts of streptomycin administered in these experiments there was no evidence of any lethal effect on the developing embryo. The low toxicity of this new agent is noteworthy.

[‡] The chart was prepared by Cpl. Fred W. Trader and photographed by the U. S. A. Signal Corps.

15213 P

Protection of Mice Against Lethal Action of Gonococcal Endotoxin by Penicillin*

C. PHILLIP MILLER AND ALDEN K. BOOR

From the Department of Medicine and the A. B. Kuppenheimer Foundation, University of Chicago

We have reported¹ that large subcutaneous doses of penicillin repeatedly administered exert a considerable degree of protection against the toxicity of sterile meningococcal endotoxin as measured by its lethal action in mice and rabbits. Neter² claimed that penicillin mixed with tetanus toxin did not protect mice from death. According to Ercoli, Lewis, and Moench³ large doses of penicillin neutralized diphtheria toxin *in vitro*, but had no effect in guinea pigs on the lethal action of

incompletely neutralized diphtheria toxin. Little other investigation has been directed toward the effect of penicillin on the action of toxic bacterial substances. Carpenter and his co-workers^{4,5,6} reported detoxification by sulfanilamide and sulfapyridine of the "toxins" of gonococcus and other bacteria.

The crude endotoxin used in these experiments was prepared from 5 strains of gonococcus grown for 18 hours on an agar medium.⁷

* Aided by a grant from the John and Mary R. Markle Foundation.

¹ Boor, Alden K., and Miller, C. Phillip, *Science*, 1945, **102**, 427.

² Neter, Edwin, *J. Inf. Dis.*, 1945, **76**, 20.

³ Ercoli, N., Lewis, M. N., and Moench, Lucille J., *J. Pharm. Exp. Ther.*, 1945, **84**, 120.

⁴ Carpenter, C. M., Hawley, P. L., and Barbour, G. M., *Science*, 1938, **88**, 530.

⁵ Barbour, Gerald M., and Carpenter, Charles M., *Am. Assn. Adv. Sci.*, Pub. No. 11, 1939, 114.

⁶ Carpenter, Charles M., and Barbour, Gerald M., *Proc. Soc. Exp. Biol. and Med.*, 1939, **41**, 354.

⁷ Boor, Alden K., *Proc. Soc. Exp. Biol. and Med.*, 1942, **50**, 22.

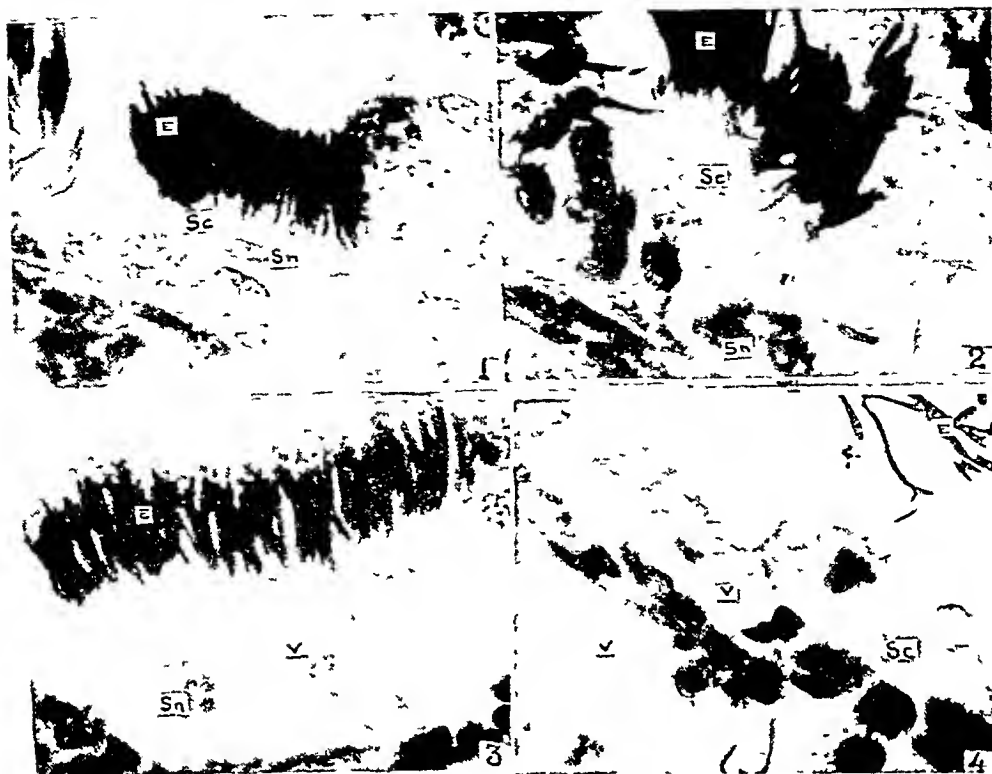


Fig 1 4 Testis of *Bufo arcnarum* Hensel Hematoxylin eosin Ob 90x, oc 6x, Cam length 60 cm Sc, Sertoli cell Sn Sertoli nucleus E spermatozoon, v vacuole

Fig 1 Normal control Spermatozoa tightly clustered upon Sertoli cells Fix Zenker formol.

Fig 2 Thirty minutes after the injection of the pituitary extract Cytoplasm of Sertoli cells is greatly increased showing a fine vacuolization and small eosinophilic granules Fix Bouin's fluid

Fig 3 One hour after injection Cytoplasm of the Sertoli cells is more hypertrophied and shows big vacuoles Fix Bouin's fluid

Fig 4 Three hours after injection Spermatozoa are completely liberated and released into the lumen Sertoli cells appear highly vacuolized and with the apical border torn (Lumen slightly retouched)

servation finding that the injection of pituitary extract produces in the testis of hibernating frog the release of the spermatozoa normally attached to the Sertoli cells. The author gives no explanation of the mechanism by which the spermatozoa are released into the lumen, but suggests that "Possibly smooth muscle fibers are involved in the expulsion of spermatozoa from the tubules (Rugh)"

In order to investigate the actual mechanism of this release toads (*Bufo arcnarum* Hensel) in sexual rest (end of summer) were injected intravenously with a total extract of 3 anterior lobes of the pituitary gland of male toads

Thirty minutes one 2, 3, and 5 hours after the injection, the testes were fixed in Bouin or in Zenker-formol fluids and also in the fixative of Champy, followed for 7 days by immersion in osmic acid at 38°C. Testes of normal control animals showed almost all mature spermatozoa clustered about their Sertoli cells (Fig 1). Thirty minutes after the injection of the pituitary extract some of the spermatozoa have been liberated. The Sertoli cells showed big increase and vacuolization of cytoplasm (Fig 2). After one hour, clear hydropic vacuoles were more conspicuous and also some acidophil droplets were

containing casein-digest and cystine. The organisms were washed off with saline, centrifuged, resuspended, and recentrifuged three times to free them from medium. The packed cells were finally suspended in water, adjusted to pH 8.0 and kept about 6 hours at room temperature and 14 hours at 4°C. Next the preparation was neutralized and sterilized by heating at 60°C for 30 minutes. Its concentration was adjusted to about 1.0% solids. All injections of endotoxin were made intraperitoneally in 18- to 20-g white mice. The toxicity of each preparation was determined by preliminary tests in batches of 5 or 6 mice for each trial dose.

The penicillin[†] used was the ordinary commercial product of several manufacturers. The mice received 8 subcutaneous injections of penicillin of 1,500 units each in 0.15 cc of water. These were usually given as follows at 90- and 45-minute intervals before the endotoxin and at 1, 3, 5, 9, 20, and 24 hours after the endotoxin. Variations in total quantity and number of doses of penicillin were tried but no improvement was found in the amount and method of administration. Mice were used in groups of 8 to 15 for each quantity of endotoxin in the control group as well as in the group receiving penicillin. Total number of deaths was determined for a period

[†] The penicillin was provided by the Office of Scientific Research and Development from supplies assigned by the Committee on Medical Research for experimental investigations recommended by the Committee on Chemotherapeutic and Other Agents of the National Research Council.

of 90 hours after injection of the endotoxin.

In most of the experiments graded doses of endotoxin were used. Representative of some of the more striking experiments are the two summarized in Table I. In each group injected with a given dose of endotoxin, more deaths occurred among the controls than among those treated by penicillin.

Some experiments showed better protection than others, but in every one fewer of the treated mice died than of the controls. The combined results of all the series, totaling 176 control mice and 210 penicillin treated, are summarized in Fig. 1, which shows the rates of death as well as final percentages.

The difference in survivals and deaths as shown on this chart may be considered significant. Whereas 76.7% of all the control mice died, only 36.5% of those receiving the penicillin treatment died.

During the first 12 hours after endotoxin injection, 18.7% of the controls and 11.4% of the penicillin-treated mice died—some as early as 3 hours. From 12 to 20 hours the highest mortality occurred—38.1% of the controls and 18% of those treated with penicillin.

Other substances such as saline and tryptic digest of casein repeatedly injected in place of the penicillin failed to influence the lethal action of the endotoxin.

Summary and Conclusions Large doses of penicillin repeatedly administered by subcutaneous injection protected a significant proportion of mice against the lethal action of a crude preparation of gonococcal endotoxin.

15214

Action of Anterior Pituitary on Sertoli Cells and on Release of Toad Spermatozoa

E. DE ROBERTIS, M. H. BURGOS, AND E. BREYTER (Introduced by Vergilio G. Foglia)
From the Institute of General Anatomy and Embryology, Faculty of Medicine, Buenos Aires,
and Institute of Biology and Experimental Medicine

Houssay and Lascano Gonzalez¹ found that in the testis of toads, in which anterior pituitary glands were implanted, spermatozoa

were released into the lumen of the seminiferous tubules. Rugh^{2,3} confirmed this ob-

¹ HOUSSAY, B. A., and LASCANO GONZALEZ, J. M., *Rev. Soc. Arg. Biol.*, 1929, **5**, 77.

² RUGH, L. R., *PROC. SOC. EXP. BIOL. AND MED.*, 1937, **36**, 418.

³ RUGH, L. R., *J. Exp. Zool.*, 1939, **80**, 81.

Additional worms will be seen in the perfusate exiting from the cut edges of the liver and from the portal vein. When no further hepatic vein perfusion yields any worms, the liver is divided into its separate lobes and these cut into smaller pieces about 3 to 4 inches square. The larger portal radicals may be perfused and additional worms obtained. By a gentle squeezing and shaking motion of the pieces of liver additional worms can be recovered. (The intact liver may be kept in saline overnight safely and the above procedure carried out

with the same results as with livers done shortly after removal from the animal's body.)

The mesentery of the large and small bowel is stripped from its attachments, cut into small pieces and placed in saline. This is allowed to stand at ice-box temperature overnight, at which time all the worms remaining in the mesenteric venous system will have escaped and seen lying on the bottom of the container.

Experiments are being carried on to determine fully the advantages of this method over other methods described.

15216

Subtilin—Antibiotic Produced by *Bacillus subtilis** II Toxicity of Subtilin to Living Embryonic Tissue,

A. J. SALLE AND GREGORY J. JANN

From the Department of Bacteriology, University of California, Los Angeles

In a previous communication¹ it was shown that subtilin, an extract prepared from *Bacillus subtilis*, was antagonistic chiefly against Gram-positive bacteria. Two notable exceptions were *Neisseria catarrhalis* and *N. gonorrhoeae*, both Gram-negative but also antagonized by subtilin. Acid-fast organisms, including *Mycobacterium tuberculosis*, and a number of pathogenic higher fungi were also found to be susceptible to the antibiotic. The agent produced a bacteriostatic action in high dilution and a germicidal effect in greater concentration.

The present communication is concerned with the toxicity of subtilin to living embryonic chick heart tissue fragments cultivated *in vitro*. The procedures followed in performing the tests were the same as those previously reported.^{2,3}

Experimental. The highest dilution of subtilin that killed embryonic chick heart tissue fragments in 10 minutes at 37°C was found

to be 1/500 (Fig. 1). The highest dilution of subtilin that killed *Staphylococcus aureus* (F.D.A. strain) under the same conditions was 1/9750 (Table I). From these results a toxicity index may be calculated.

The toxicity index may be defined as the ratio of the highest dilution of subtilin killing embryonic chick heart tissue after an exposure period of 10 minutes at 37°C to the highest dilution killing *Staphylococcus aureus* under the same conditions. The results give a toxicity index of $500/9750 = 0.05$ (Table II). Under the conditions of the test, subtilin was found to be 20 times more toxic to *Staphylococcus aureus* than to embryonic chick heart tissue, a remarkably low figure. Theoretically, the smaller the toxicity index, the more nearly perfect the germicide.

Untage. The methods used for expressing the potencies of the various antibiotics are, in general, quite complicated and confusing. In order to simplify the method of designating

* This investigation was aided by a grant from Eli Lilly and Company, Indianapolis, Ind.

† The subtilin preparation used in these experiments was kindly supplied by the Western Region of Research Laboratory, Albany, Calif.

¹ Salle, A. J., and Jann, Gregory, J., *Proc. Soc.*

EXP. BIOL. AND MED., 1945, **60**, 60.

² Salle, A. J., McOmie, W. A., Sheehmeister, I. L., and Foord, D. C., *Proc. Soc. EXP. BIOL. AND MED.*, 1938, **37**, 694.

³ Salle, A. J., McOmie, W. A., Sheehmeister, I. L., and Foord, D. C., *J. Bact.*, 1939, **37**, 639.

found (Fig 3) The vacuoles have the tendency to aggregate and seem to push the spermatozoa away from the tubule wall Three hours after the injection almost all mature spermatozoa have been released Most of the Sertoli cells appear enormously vacuolized and with the apical border completely irregular, due to the rupture of some vacuoles (Fig 4) During this process of vacuolization, the

Golgi apparatus appears fragmented and situated close to the vacuoles

The observations recorded here show that possibly the release of the spermatozoa of the toad, under the influence of the pituitary gland, is due to the intense changes produced in the Sertoli cells, which lead to the rupture and disintegration of the apical part of their cytoplasm

15215 P

A Method for Removal of Adult *S. mansoni* from Experimentally Infected Rabbits

J LEONARD BRANDT AND E P FINCH (Introduced by J Oliver Gonzalez)

From the Department of Medical Zoology, School of Tropical Medicine,* San Juan, Puerto Rico

Herein is described a method for the removal of adult *S. mansoni* worms from experimentally infected rabbits The method has been used with good results in rabbits although there is no apparent reason why it could not be applied to other experimental animals susceptible to schistosomiasis

The animal is tied to a board and a large dose (100 mg) of Heparin (Abbott—10 mg per cc) in 30 to 40 cc of physiological saline is injected very slowly into the heart or intravenously Between 15 to 30 minutes are allowed to elapse at which time the animal is sacrificed in any desired fashion, the abdomen opened and the portal vein exposed An 18-gauge needle with attached 50-cc syringe is inserted into the portal vein with the point of the needle directed towards the liver The heparinized blood in the portal vein is aspirated while the syringe and needle are rotated to direct the bevel of the needle toward the worms which will be seen flowing into the syringe with the blood About 15 to 20 cc can thus be aspirated before the portal vein collapses

An additional 10 to 15 cc of blood can be aspirated by exerting a gentle squeezing pressure on the lobes of the liver—this will force more blood into the portal vein, and the liver will be seen to blanch slightly The syringe is disconnected from the needle and put aside without fear of the blood clotting A clean dry syringe may be attached to the needle and additional aspirations carried out in the manner described

If it is desired to keep the worms alive, the aspirated blood is poured into a solution of 0.5% saponin in physiological salt solution (0.85%)—this will lysis the blood cells but have no apparent effect on the worms If it is not necessary to keep the worms alive, the aspirated blood is poured into a large volume of tap water—this will lysis the blood cells, and the dead worms will sink to the bottom of the vessel

When no further aspirations can be done the needle is removed from the portal vein The liver is removed as a unit by cutting and clamping the hepatic vein, portal vein, surrounding ligaments and adhesions and kept in saline for further recovery of worms It is then placed on a dissecting board and the thin tips of the various liver lobes are cut off about 1/4 inch from their edges Using a 50-cc syringe, physiological saline is perfused through the liver by injecting the hepatic vein

* The authors wish to express their gratitude to Dr P Morales Otero, Director, and to Dr Jose Oliver Gonzalez of the School of Tropical Medicine for their advice and encouragement during this work

Additional worms will be seen in the perfusate exiting from the cut edges of the liver and from the portal vein. When no further hepatic vein perfusion yields any worms, the liver is divided into its separate lobes and these cut into smaller pieces about 3 to 4 inches square. The larger portal radicals may be perfused and additional worms obtained. By a gentle squeezing and shaking motion of the pieces of liver additional worms can be recovered. (The intact liver may be kept in saline overnight safely and the above procedure carried out

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15216

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EXP. BIOL. AND MED., 1945, 60, 60.

² Salle, A. J., McOmie, W. A., Sheehmeister, I. L., and Foord, D. C., Proc. Soc. Exp. Biol. and Med., 1938, 37, 694.

³ Salle, A. J., McOmie, W. A., Sheehmeister, I. L., and Foord, D. C., J. Bact., 1939, 37, 639.

found (Fig 3) The vacuoles have the tendency to aggregate and seem to push the spermatozoa away from the tubule wall Three hours after the injection almost all mature spermatozoa have been released Most of the Sertoli cells appear enormously vacuolized and with the apical border completely irregular, due to the rupture of some vacuoles (Fig 4) During this process of vacuolization, the

Golgi apparatus appears fragmented and situated close to the vacuoles

The observations recorded here show that possibly the release of the spermatozoa of the toad, under the influence of the pituitary gland, is due to the intense changes produced in the Sertoli cells, which lead to the rupture and disintegration of the apical part of their cytoplasm

15215 P

A Method for Removal of Adult *S. mansoni* from Experimentally Infected Rabbits

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Herein is described a method for the removal of adult *S. mansoni* worms from experimentally infected rabbits The method has been used with good results in rabbits although there is no apparent reason why it could not be applied to other experimental animals susceptible to schistosomiasis

The animal is tied to a board and a large dose (100 mg) of Heparin (Abbott—10 mg per cc) in 30 to 40 cc of physiological saline is injected very slowly into the heart or intravenously Between 15 to 30 minutes are allowed to elapse at which time the animal is sacrificed in any desired fashion, the abdomen opened and the portal vein exposed An 18-gauge needle with attached 50-cc syringe is inserted into the portal vein with the point of the needle directed towards the liver The heparinized blood in the portal vein is aspirated while the syringe and needle are rotated to direct the bevel of the needle toward the worms which will be seen flowing into the syringe with the blood About 15 to 20 cc can thus be aspirated before the portal vein collapses

An additional 10 to 15 cc of blood can be aspirated by exerting a gentle squeezing pressure on the lobes of the liver—this will force more blood into the portal vein, and the liver will be seen to blanch slightly The syringe is disconnected from the needle and put aside without fear of the blood clotting A clean dry syringe may be attached to the needle and additional aspirations carried out in the manner described

If it is desired to keep the worms alive, the aspirated blood is poured into a solution of 0.5% saponin in physiological salt solution (0.85%)—this will lysis the blood cells but have no apparent effect on the worms If it is not necessary to keep the worms alive, the aspirated blood is poured into a large volume of tap water—this will lysis the blood cells, and the dead worms will sink to the bottom of the vessel

When no further aspirations can be done the needle is removed from the portal vein The liver is removed as a unit by cutting and clamping the hepatic vein, portal vein, surrounding ligaments and adhesions and kept in saline for further recovery of worms It is then placed on a dissecting board and the thin lips of the various liver lobes are cut off about $\frac{1}{4}$ inch from their edges Using a 50-cc syringe, physiological saline is perfused through the liver by injecting the hepatic vein

* The authors wish to express their gratitude to Dr P Morales Otero, Director, and to Dr Jose Oliver Gonzalez of the School of Tropical Medicine for their advice and encouragement during this work

Amyloidosis in Hamsters with Leishmaniasis^{*}

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During the course of an investigation of chemotherapeutic agents in experimental leishmaniasis, the development of anasarca in infected animals was first noted by R O Greep in the early stages of the experimental program in the Squibb Institute in New Brunswick, New Jersey. Later, routine histopathological examination as well as chemical investigation of the blood revealed the widespread presence of amyloid deposits and a reversal of the albumin-globulin ratio of the plasma proteins. In human kala azar, the presence of generalized edema is not infrequent and the alteration of the serum albumin-globulin ratio is characteristic, however, neither in clinical nor in experimental leishmaniasis have we been able to find a report of amyloidosis.¹ Nevertheless, it seems likely that the hamsters described in the note just published by Goodwin² suffered from amyloidosis. In his animals, as in ours, infection by *Leishmania* might be followed by edema, nephritis, proteinuria, and depression of the level of plasma proteins. The present investigation was undertaken to correlate the development of amyloidosis and the changes in the distribution of proteins in the plasma during the progress of leishmaniasis in hamsters. From the experimental results it was hoped that answers to two questions would emerge: (1) What is the temporal relationship between the deposition of amyloid and the alteration of the plasma protein concentration, and (2) Alterations in which organ, the liver or the kidney, are chiefly responsible for the reduction of

the plasma albumin?

Materials and Methods A single large group of hamsters (*Cricetus auratus*), six weeks old, served as the experimental animals. They were maintained on pellets of Rockland rat diet ("D" free) supplemented with lettuce, carrots, and whole wheat bread. The animals were divided into two groups at random and at the beginning of the experiment the animals of one group were inoculated intraperitoneally with 20 mg of spleen in a saline emulsion which was obtained from 2 hamsters heavily infected with *L. donovani* (Khartoum strain). The other group served as the uninfected control. At approximately two-week intervals, 5 representatives each from the control and the infected groups were placed in individual metabolism cages and the urine was collected for 24 hours.

Immediately after the termination of the urine collection, the animals were anesthetized with intraperitoneal evipal sodium (140 mg/kg body weight) and heparinized blood samples were obtained by heart puncture. The animals were then sacrificed, the tissues removed for histological examination were fixed either in Zenker's solution without acetic acid or in Vandegriff's dehydrating fixative.

Owing to the small size of the plasma sample for the determination of non-protein nitrogen, it was necessary to determine this nitrogen fraction by a Nessler procedure which was adopted for all nitrogen determinations. The solutions of protein or non-protein nitrogen were first digested in Pyrex Nessler tubes in the presence of 1 ml of 19 N sulfuric acid, oxidation was completed by a drop or two of 30% hydrogen peroxide. The digested material was diluted with water to 35 ml and 15 ml of Nessler's reagent made according to the directions of Folin³ were added. The final

* This work has been carried out under contract, recommended by the Committee on Medical Research, between the Office of Scientific Research and Development and Columbia University.

¹ Stitt's *Diagnosis, Prevention, and Treatment of Tropical Diseases* edited by R P Strong, Philadelphia 1943.

² Goodwin, L. G., *Nature*, 1945, **156**, 476.

³ Folin, O., *Laboratory Manual of Biological Chemistry* New York, 1934.

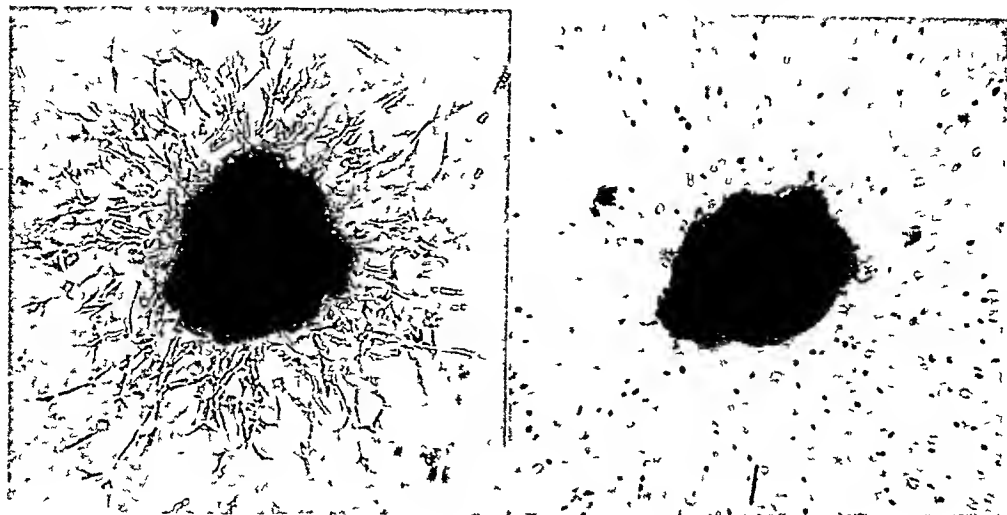


FIG 1

Left, fragment of chick heart tissue exposed to a sublethal concentration of subtilin. Note new growth of fibroblasts. Right, fragment exposed to a lethal concentration of subtilin.

TABLE I

Killing Dilutions of Subtilin for Chick Heart Tissue Fragments and *Staphylococcus aureus*

Tissue fragments		<i>Staphylococcus aureus</i>	
Dilution	Growth	Dilution	Growth
1:100	—	1:9000	—
1:200	—	1:9250	—
1:300	—	1:9500	—
1:400	—	1:9750	—
1:500	—	1:10,000	+
1:600	+	1:10,250	+
1:700	+	1:10,500	+
1:800	+	1:10,750	+
Control	+	Control	+

TABLE II
Toxicity Index of Subtilin

Killing dilution		Toxicity index A/B
Tissue (A)	<i>Staphylococcus aureus</i> (B)	
1:500	1:9750	0.05

the strength of subtilin as much as possible, a unit is defined as that amount present in 1 cc of the highest dilution (expressed in mg) capable of killing *Staphylococcus aureus* in 10 minutes at 37°C (F D A phenol coefficient method). For example, 5 cc of a 1:9750 dilution of the preparation used, when mixed with 0.5 cc of a nutrient broth culture of

Staphylococcus aureus, completely destroyed all organisms in 10 minutes at 37°C. One cc of this dilution contains approximately 0.1 mg of this preparation of subtilin. Therefore, 0.1 mg contains 1 unit of subtilin. The weight of subtilin containing 1 unit will vary depending upon the purity of the product.

The 24-hour culture of *Staphylococcus aureus* was standardized in a photoelectric colorimeter to contain the same density as a No. 7 McFarland Nephelometer Standard.

Summary. Subtilin, an antibiotic extracted from *Bacillus subtilis*, was found to be antagonistic chiefly against Gram-positive organisms, including *Mycobacterium tuberculosis* and other acid-fast bacteria. Two notable exceptions were *Neisseria catarrhalis* and *N. gonorrhoeae*, both Gram-negative but also antagonized by subtilin. The antibiotic showed an extremely low toxicity for embryonic chick heart tissue fragments cultivated *in vitro*. Under the conditions of the test, subtilin was approximately 20 times more toxic to *Staphylococcus aureus* than to chick heart tissue, a remarkably low tissue toxicity. A unit of subtilin is defined as the amount contained in 1 cc of the highest dilution capable of killing *Staphylococcus aureus* in 10 minutes at 37°C (F D A phenol coefficient method).

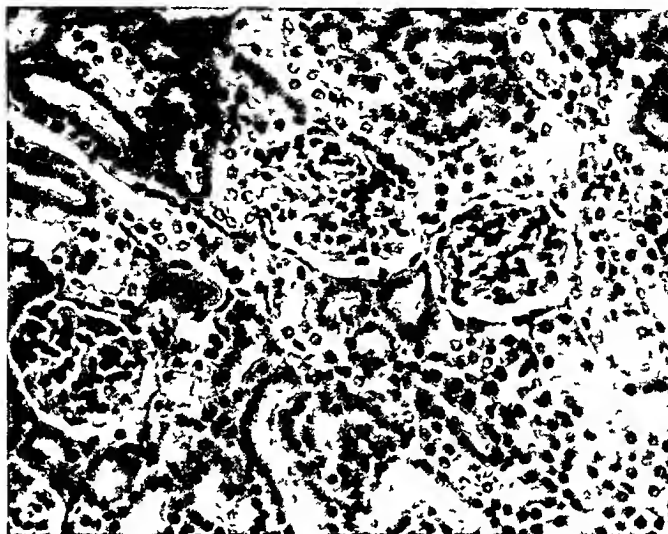


FIG 1
Kidney of normal hamster Hematoxylin and eosin $\times 275$

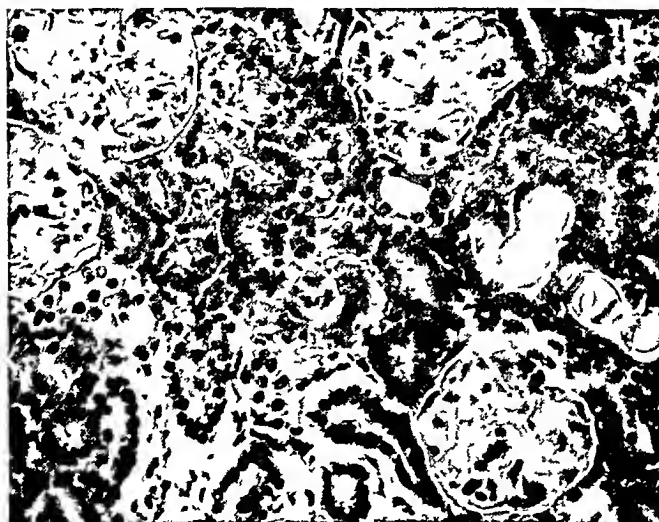


FIG 2
Kidney of hamster with amyloidosis 77 days after inoculation of *L. donovani*. Note extensive replacement of glomeruli by amyloid deposited between endothelium and epithelium. The tubular casts are also typical. Amyloid deposited about the tubules and extending into the interstitial tissue is an infrequent finding illustrated in this section. Hematoxylin and eosin $\times 275$

kidneys was deposited in the glomeruli and appeared to replace three-fourths or more of this structure in the late stages of the disease (*Leishmania* were rarely found in the kidney). The glomerular amyloid accumulated between

the capillary endothelium and the capillary basement membrane, it was never found external to the latter. Trivial deposits of amyloid were observed between the epithelium and the basement membrane of the tubular epithelium.

TABLE I
Plasma Proteins, Amyloidosis and Edema in Hamsters with Experimental Leishmaniasis

Days after infection		Amount of amyloid in											No of animals
		Albumin (g%)	S D	Globulin (g%)	S D	A/G Ratio	S D	Edema	Kidneys (glomeruli)	Adrenal cortex	Spleen	Liver	
18	Normal	2.76	0.16	2.06	0.17	1.35	0.18	0	0	0	0	0	3
	Infected	2.12	0.62	3.06	0.61	0.75	0.38	0	0	0	0	0	5
32	Normal	3.09	0.35	2.19	0.43	1.45	0.31	0	M 1/5	M 1/5	M 1/5	M 2/5	5
	Infected	3.07	0.45	2.76	0.23	1.11	0.20	0	M 1/5	M 2/5	M 1/5	M 1/5	5
46	Normal	2.34	0.33	2.56	0.21	0.92	0.16	0	0	0	0	0	5
	Infected	1.09	0.66	2.72	0.40	0.40	0.22	+2/5	+ + +5/5	+ +5/5	+4/5	+4/5	5
60	Normal	3.17	0.17	1.98	0.19	1.60	0.17	0	M 1/5	0	M 1/5	M 1/5	5
	Infected	0.48	0.21	2.22	0.58	0.22	0.07	+ + + +3/3	+ + + +3/3	+ + + +3/3	+3/3	+3/3	3
77	Normal	2.46	0.26	2.26	0.24	1.11	0.06	0	0	0	0	0	3
	Infected	0.61	0.17	2.34	0.29	0.26	0.05	+ + + +4/4	+ + + +4/4	+ + +4/4	+4/4	+4/4	4

* M = Minimal

* M = Mummified

estimates were made in a Beckman photoelectric spectrophotometer at a wave length of 400 mμ. The useful range of amounts of nitrogen per sample was about 0.02 to 0.15 mg. The blank solution was similarly made from reagents alone, with known amounts of nitrogen added as a solution of pure $(\text{NH}_4)_2\text{SO}_4$, there was a straight-line relationship between optical density and nitrogen-concentration. The method of Howe⁴ was employed to separate plasma globulin and thus to determine the concentration of albumin and globulin in plasma. The non-protein nitrogen of urine and plasma was estimated in filtrates containing, after suitable dilution with water, 5% trichloroacetic acid. The histological examinations were made independently by one of us (WJP) and it was not until the experiment was completed that the results of the pathological examination and the chemical changes in the blood were compared. Paraffin sections of the tissues were made and stained with hematoxylin and eosin, Giemsa's stain, or Congo Red which was sometimes combined with Wilder's reticulum stain.

Results The principal gross pathological findings in infected hamsters were splenomegaly, enlarged lymph nodes, pale kidneys and anasarca. The gross renal change and the anasarca were not evident until 46-60 days after inoculation. There were no significant gross changes in the tissues of the normal controls. The microscopic alterations owing to leishmaniasis conformed to the description of other authors with the exception of the presence of amyloidosis which was found in all hamsters which had been inoculated with *L. donovani* at least 46 days previously. Small amyloid deposits were found in 3 of the 21 control animals, the quantities present were minimal.

The incidence of the amyloidosis is summarized with the other data in Table I. Amyloid was deposited chiefly in the renal glomeruli, the adrenal cortex, the spleen and the liver. Its appearance and distribution were characteristic of "secondary" amyloidosis of man and animals. No intracellular amyloid was identified. The amyloid of the

⁴ Howe, P. L., *J. Biol. Chem.*, 1921, **49**, 93

continues precipitously. In the next interval of 17 days there is no further change in the plasma albumin concentration of the infected animals. During the entire time when the striking fall in plasma albumin concentration is occurring, there is no significant deviation of the plasma globulin level in the infected animals as compared with the normal group. Amyloidosis is maximal at the 60th day and the distribution of amyloid is at all times more widespread in the kidney (glomeruli) than in the liver. The presence of edema is correlated with the hypoalbuminemia as one would expect.

The plasma non-protein nitrogen concentration in the infected animals was not significantly different from the control in all the experiments except at 60 days. In this experiment the non-protein nitrogen concentration of the infected group was clearly above the level in the control animals.

Fig 3 presents the results of the plasma protein studies graphically, and, in addition, summarizes the quantitative estimation of proteinuria. It can be seen that at no time did the control groups show any protein in the urine. In the infected animals, proteinuria made its first appearance after 46 days, when the first observation of hypoalbuminemia was made. Proteinuria was always observed subsequently. The available evidence suggests that the urinary protein was albumin, none could be precipitated in the presence of 1.5 M sodium sulfate.

Discussion In another series of hamsters attempts to inoculate one strain of *L. infantum* by intraperitoneally-injected splenic emulsion did not produce infection or recognizable edema within the following 6-8 weeks. For this reason, normal spleen was not administered intraperitoneally to the control animals.

In all the experiments, the percentage of globulin in the plasma of the infected animals was higher than that of the normals, however, no statistical significance can be attached to these differences. (Because of the high percentage of globulin in the plasma of the infected hamsters of the first experiment, the A/G ratio was reduced.) There can be no doubt that in hamsters infected with

Leishmania donovani 46 days or longer, there was a significant fall in the plasma albumin and in the A/G ratio in comparison with corresponding control animals. The highest probability that such a change could occur by chance was less than 0.01 (comparison of albumin-concentrations of experiment 3). In all other comparisons of plasma albumin percentage in plasma or of the A/G ratio, the probability of chance occurrence was even lower—especially in the last 2 experiments. Albuminuria in the infected hamsters was first detected 46 days after infection when a significant lowering of plasma albumin was also first observed. The small amount of albumin excreted in the urine 60 days after infection is to be explained by the small quantity of urine excreted owing to excessively hot weather. All the protein in the urine may have been albumin since no globulin could be precipitated by warm sodium sulfate solution according to Howe's plasma method, however, no dogmatic statement in this regard can be made since the experimental conditions are seriously altered when urine instead of plasma is the protein solvent and the concentration of protein present is different from that of plasma.

The animals infected 46 days or longer, in contrast with the controls, had hypoalbuminemia, proteinuria, edema (characterized by anasarca in the last 2 experiments) and deposits of amyloid in the kidneys, adrenal cortex, spleen and liver. These changes occurred 46 days after infection. It is concluded that the primary change, amyloid deposition in the renal glomeruli followed later by a similar but slight change in the tubules, led to excessive loss of albumin in the urine with an associated reduction of plasma albumin concentration and lowering of the colloid osmotic pressure of plasma so that excessive amounts of fluid escaped into the tissues. It is not believed that failure of the synthesis of plasma albumin was responsible to any important extent for the lowering of the percentage of this protein in the plasma.

Summary Forty-six days after successful inoculation of *L. donovani* into hamsters, there appeared edema associated with amyloidosis. There was marked anasarca at 60

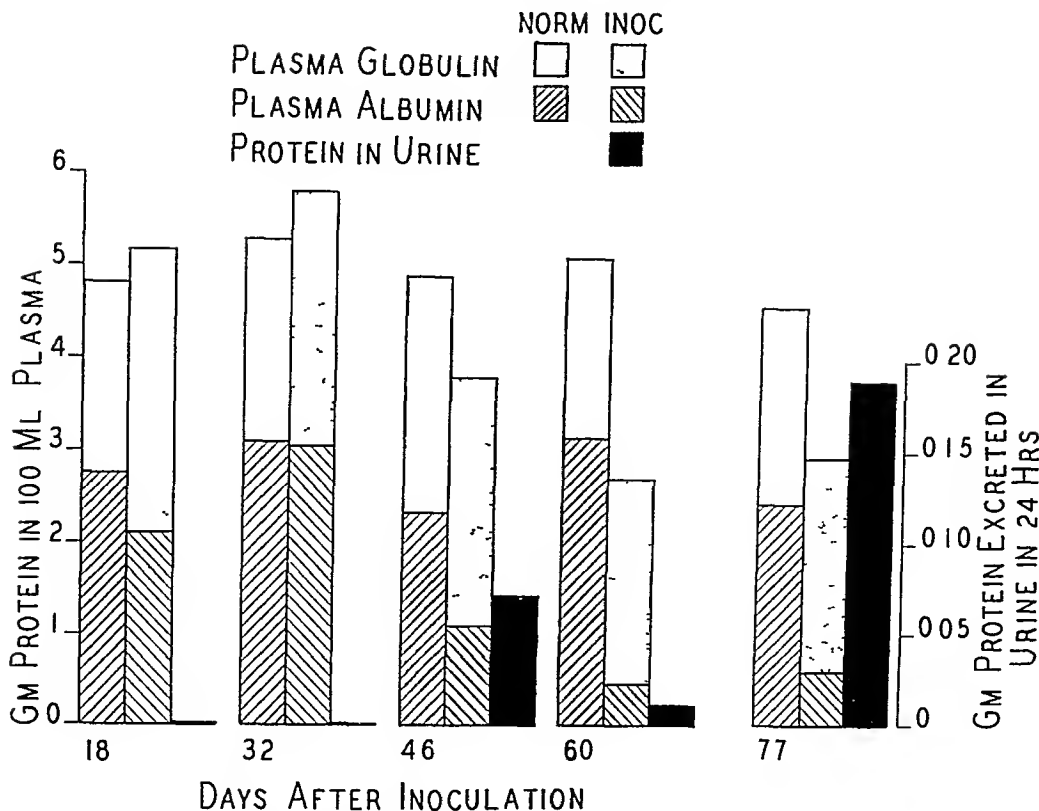


Fig 3

The concentration of plasma proteins in normal and infected hamsters. Also shown is the total urinary protein excretion in infected hamsters.

lum Hyaline droplets or casts could be found in the tubules of the kidneys with severe amyloidosis (Fig 1 and 2). In the cortex of the adrenals there was likewise bilateral deposition of amyloid which in some cases replaced more than half of the normal tissue. The early deposits in the adrenal cortex were clearly between the capillary endothelium and the basement membrane. The amyloid of the spleen was deposited about the widely separated malpighian corpuscles and, to a less extent, in the ring of pulp surrounding the spherical masses of phagocytic cells containing parasites. In the liver, amyloid was first found beneath the endothelium of the hepatic and portal veins from which it occasionally spread into the parenchyma. Deposits sometimes occurred around parasite-filled phagocytic cells. The amounts of amyloid in spleen and liver were always small.

Table 1 also presents the quantitative data on the plasma albumin and globulin and the A/G ratio at intervals after the inoculation of the animals. The standard deviations are also tabulated to indicate the range of the observations and their distribution about the mean. It is to be noted that 46 days after the infection there is a significant decrease in the plasma albumin concentration of the infected animals as compared with the controls. The globulin concentration is not demonstrably different in the two groups and as a result the A/G ratio is significantly lower in the infected animals. It is also to be noted that at this time the deposition of significant amounts of amyloid makes its first appearance and that it is found to be most abundant in the glomeruli of the kidney. Between the 46th day and the 60th day the decrease in plasma albumin concentration in the infected animals

TABLE I
Precipitation of Cubohydrate Derived from *Listeria monocytogenes* in Antiserum of Different Strains

Antiserum against strains of <i>Listeria monocytogenes</i>	Source of strain	Cubohydrates from strain											
		Strain X				Murray No 204				R 1			
		0.4	0.1	0.02	cc	0.4	0.1	0.02	cc	0.4	0.1	0.02	cc
Strain X	Unknown	+	+	+	cc	+	+	+	cc	+	+	+	cc
R 1	Rabbit	+	+	+	cc	+	+	+	cc	+	+	+	cc
Gibson	"	+	+	+	cc	+	+	+	cc	+	+	+	cc
Nyfeldt	"	+	+	+	cc	+	+	+	cc	+	+	+	cc
Juhnelle	"	+	+	+	cc	+	+	+	cc	+	+	+	cc
Schultz	"	+	+	+	cc	+	+	+	cc	+	+	+	cc
Murray No 204	Rabbit	+	+	+	cc	+	+	+	cc	+	+	+	cc
Bovine 7647	Cow	+	+	+	cc	+	+	+	cc	+	+	+	cc
S 1	Sheep	+	+	+	cc	+	+	+	cc	+	+	+	cc
H 14	Man	+	+	+	cc	+	+	+	cc	+	+	+	cc
B 8	Cow	+	+	+	cc	+	+	+	cc	+	+	+	cc
D 82 N	Cattle	+	+	+	cc	+	+	+	cc	+	+	+	cc
G 1	Goat	+	+	+	cc	+	+	+	cc	+	+	+	cc
Burn	Man	+	+	+	cc	+	+	+	cc	+	+	+	cc
Seastone	Fowl	+	+	+	cc	+	+	+	cc	+	+	+	cc

Antiserum against strains of <i>Listeria monocytogenes</i>	Source of strain	Cubohydrates from strain											
		Nyfeldt				Juhnelle				Schultz			
		0.4	0.1	0.02	cc	0.4	0.1	0.02	cc	0.4	0.1	0.02	cc
Strain X	Unknown	+	+	+	cc	+	+	+	cc	+	+	+	cc
R 1	Rabbit	+	+	+	cc	+	+	+	cc	+	+	+	cc
Gibson	"	+	+	+	cc	+	+	+	cc	+	+	+	cc
Nyfeldt	"	+	+	+	cc	+	+	+	cc	+	+	+	cc
Juhnelle	"	+	+	+	cc	+	+	+	cc	+	+	+	cc
Schultz	"	+	+	+	cc	+	+	+	cc	+	+	+	cc
Murray No 204	Rabbit	+	+	+	cc	+	+	+	cc	+	+	+	cc
Bovine 7647	Cow	+	+	+	cc	+	+	+	cc	+	+	+	cc
S 1	Sheep	+	+	+	cc	+	+	+	cc	+	+	+	cc
H 14	Man	+	+	+	cc	+	+	+	cc	+	+	+	cc
B 8	Cow	+	+	+	cc	+	+	+	cc	+	+	+	cc
D 82 N	Cattle	+	+	+	cc	+	+	+	cc	+	+	+	cc
G 1	Goat	+	+	+	cc	+	+	+	cc	+	+	+	cc
Burn	Man	+	+	+	cc	+	+	+	cc	+	+	+	cc
Seastone	Fowl	+	+	+	cc	+	+	+	cc	+	+	+	cc

and 77 days after infection with extensive deposition of amyloid in the glomeruli and the adrenal cortex. The hypoalbuminemia, which coincided with the edema and a protemuria, is believed to have been caused by the impairment of glomerular function.

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Occurrence of Two Immunological Groups Within the Genus *Listeria* Studies Based upon Precipitation Reactions

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Thus far, attempts to establish the serological reactions and antigenic structure of microorganisms of the genus *Listeria* have been based upon agglutination and agglutinin absorption studies. Paterson¹ concluded that the antigenic structure permitted division of the genus into 4 types. He stated, "the bacteriological types do not bear any relation to the zoological species of the host or to the geographical distribution of the places of isolation." Schultz, Terry, Brice and Gebhardt² on the other hand found that among 11 different strains of *Listeria monocytogenes* only 2 serological groups occurred. The strains were derived from various animal sources, including man. On the basis of the agglutination reaction Julianelle and Pons³ divided 8 strains into 2 types which they designated Type I and Type II. Type I represented strains isolated from rodent animals and Type II from ruminants. Strains isolated from the disease in man fell into one or the other of the types. Julianelle⁴ later observed that filtrates from broth cultures gave a precipitate only with their type specific immune sera. The precipitate was suggestive of that seen in the precipitation of specific polysaccharides in the presence of immune serum. The purpose of this paper is to describe the precipitation reactions of 16 strains

of *Listeria monocytogenes*.

Material and Methods The precipitinogen was a polysaccharide extracted from the bacterial cell according to the method of Fuller⁵. Chemically, the carbohydrate fractions were characterized by strongly positive Molisch reactions, and Fehling's solution was not reduced. The biuret and ninhydrin reactions were negative. For the preparation of polysaccharide and the immunization of rabbits bacteria in the smooth phase were selected. All cultures were grown in tryptic digest broth containing 0.1% dextrose and incubated 18 hours at room temperature (25-30° C).

Vaccines were prepared from broth cultures, which were centrifuged and the bacterial sediment resuspended in normal salt solution containing 0.2% formalin. The vaccine was injected intravenously into adult rabbits until the precipitin titer of the serum was sufficient to give a marked, compact-disc precipitate in the presence of the specific carbohydrate within a period of 15 minutes at room temperature. The animals were then bled to death from the heart. Two rabbits were immunized against each strain and the sera from the final bleedings pooled. Control sera were taken from all rabbits before the administration of vaccine and tested for the presence of precipitins to the carbohydrate of that strain of organism to be used for the production of immune serum. In no instance were normal precipitins demonstrated.

Precipitation as determined by the ring test using equal amounts of carbohydrate and immune serum was indicative, however, it

¹ Paterson, J. S., *J. Path. and Bact.*, 1940, **51**, 424.

² Schultz, E. W., Terry, M. C., Brice, A. T., Jr., and Gebhardt, L. P., *Proc. Soc. Exp. Biol. and Med.*, 1938, **38**, 605.

³ Julianelle, L. A., and Pons, C. A., *Proc. Soc. Exp. Biol. and Med.*, 1939, **40**, 364.

⁴ Julianelle, L. A., *J. Bact.*, 1941, **42**, 367.

⁵ Fuller, A. T., *Brit. J. Exp. Path.*, 1938, **19**, 130.

seemed advisable in this case to use tubes containing 3 dilutions of the specific soluble substance. Subsequently, precipitation tests were carried out in 10mm x 100mm tubes using 0.4 cc, 0.1 cc, and 0.02 cc amounts of specific soluble substance for each strain of *L. monocytogenes*. Sufficient normal salt solution was added to the tubes containing 0.1 cc and 0.02 cc to bring the final volume in the tube to 0.4 cc. The carbohydrate dilution in each tube was then layered with 0.2 cc of immune serum and the tubes allowed to stand at room temperature for 30 minutes. At the end of this time the presence or absence of ring formation was noted. The tubes were shaken gently and then incubated for 2 hours in a water bath at 37° C. Final readings were taken after storage in the refrigerator overnight at 10° C and recorded as +, ++, +++, and ++++. In the case of the +++ and ++++ reactions the ring tests were definite at the end of 30 minutes and usually the precipitate was visible within 10 minutes after adding the serum.

Results Table I gives the results of the precipitation reactions among 16 strains of *Listeria monocytogenes* studied. On the basis of the precipitation of the carbohydrates in antibacterial sera the strains fell into 2 rather distinct immunological groups, a rodent group and a ruminant group. The existence of 2 immunological groups has been suggested previously by the agglutination reactions. Of the strains used in this study the "Virginia" is possibly the most recent, having been isolated in 1944 from the brain at necropsy of a case of encephalitis occurring in a cow. It was readily identified with the ruminant group by means of the precipitin test. From the table it may be seen that the antibacterial sera of strains S-1 and H-14 show some cross precipitation with the carbohydrates of the rodent group, but the reactions do not appear marked and are not regarded as significant. Antibacterial serum against the Nyfeldt strain precipitated the carbohydrates of the rodent group; however, the Nyfeldt carbohydrate, although reacting with its homologous immune serum, failed to precipitate with the antibacterial sera prepared against the rodent strains. This phenomenon remained unexplained.

Polysaccharides prepared from 8 unrelated species of bacteria failed, in all but two instances, to precipitate with antibacterial sera of *Listeria monocytogenes*. Nyfeldt and Gibson antisera precipitated the carbohydrate of hemolytic *Staphylococcus aureus*. The carbohydrates of *Listeria monocytogenes* did not precipitate in antistreptococcal antityphoid, and antidyentery (Flexner) sera. It is of interest that among the strains from human infections a few are identified as belonging to the rodent group while others fall into the ruminant group.

Discussion The origin of human infections has remained an enigma; however, it seems entirely possible that they may be derived from some animal source. Carey⁶ considered this possibility in dealing with a case of acute cerebrospinal meningitis caused by *L. monocytogenes*. As far as could be determined, in this particular case, the child had had no known contact with rabbits or other animals. It is interesting to speculate that further classification of human infection might follow if the specific soluble substance were to be demonstrated in the cerebrospinal fluid from cases of *Listeria meningitis* and encephalitis. Direct precipitation of the soluble substance from the spinal fluid in the presence of specific immune serum might readily occur. It is suggested that such a procedure if established would possibly prove to be of diagnostic value.

Summary Polysaccharides have been isolated from *Listeria monocytogenes* which precipitate in the presence of their specific immune sera. The immunological studies presented indicate the existence of 2 distinct animal groups, namely a rodent group and a ruminant group, thus corroborating the classification suggested by Schultz⁷ and Juhanelle.⁸ The precipitin test affords a simplified technique for the identification and immunological differentiation of the members of the genus *Listeria*.

⁶ Carey, B. W., Jr., *J. Pediatr.* 1936, 8, 626.

I wish to thank Drs. E. W. Schultz and M. L. Robbins for the strains of *L. monocytogenes* which they so kindly made available. Also Dr. R. D. Hitch for the strain which was isolated in Virginia in 1944 and which for convenience has been referred to as the Virginia strain.

TABLE I (Cont.)

Carbohydrates from strain

Antiserum against strains of <i>L. monocytogenes</i>	Source of strain	B 8			S 1			G 1			H 14		
		0.4	0.1	0.02	0.4	0.1	0.02	0.4	0.1	0.02	0.4	0.1	0.02
		cc	cc	cc	cc	cc	cc	cc	cc	cc	cc	cc	cc
Strain X	Unknown												
R 1	Rabbit	—	—	—	—	—	—	—	—	—	—	—	—
Gibson	Man	—	—	—	—	—	—	—	—	—	—	—	—
Nyfeldt	"	—	—	—	—	—	—	—	—	—	—	—	—
Jahnelke	"	—	—	—	—	—	—	—	—	—	—	—	—
Schultz	"	—	—	—	—	—	—	—	—	—	—	—	—
Murray No 204	Rabbit	—	—	—	—	—	—	—	—	—	—	—	—
Bovine 7647	Cow	—	—	—	—	—	—	—	—	—	—	—	—
S 1	Sheep	+	+	—	+	+	+	+	+	+	+	+	+
H 14	Man	+	+	—	+	+	+	+	+	+	+	+	+
B 8	Cow	+	+	—	+	+	+	+	+	+	+	+	+
D 82 N	Cattle	+	+	—	+	+	+	+	+	+	+	+	+
G 1	Goat	+	+	—	+	+	+	+	+	+	+	+	+
Burn	Man	—	—	—	—	—	—	—	—	—	—	—	—
Seistone	Fowl	+	+	—	+	+	+	+	+	+	+	+	+

Carbohydrates from strain

Antiserum against strains of <i>L. monocytogenes</i>	Source of strain	Burn			D 82 N			Virgin			Seistone		
		0.4	0.1	0.02	0.4	0.1	0.02	0.4	0.1	0.02	0.4	0.1	0.02
		cc	cc	cc	cc	cc	cc	cc	cc	cc	cc	cc	cc
Strain Y	Unknown												
R 1	Rabbit	—	—	—	—	—	—	—	—	—	—	—	—
Gibson	Man	—	—	—	—	—	—	—	—	—	—	—	—
Nyfeldt	"	—	—	—	—	—	—	—	—	—	—	—	—
Jahnelke	"	—	—	—	—	—	—	—	—	—	—	—	—
Schultz	"	—	—	—	—	—	—	—	—	—	—	—	—
Murray No 204	Rabbit	—	—	—	—	—	—	—	—	—	—	—	—
Bovine 7647	Cow	—	—	—	—	—	—	—	—	—	—	—	—
S 1	Sheep	+	+	+	+	+	+	+	+	+	+	+	+
H 14	Man	+	+	+	+	+	+	+	+	+	+	+	+
B 8	Cow	+	+	+	+	+	+	+	+	+	+	+	+
D 82 N	Cattle	+	+	+	+	+	+	+	+	+	+	+	+
G 1	Goat	+	+	+	+	+	+	+	+	+	+	+	+
Burn	Man	—	—	—	—	—	—	—	—	—	—	—	—
Seistone	Fowl	+	+	+	+	+	+	+	+	+	+	+	+

— = No visible precipitate, + to +++ = Various degrees of visible precipitation

TABLE I
Showing Loss of Weight of Tibialis Muscle in Arthritis

Animal No	Days of arthritis	Wt in g		% difference
		N	A	
1	4	4,930	4,620	- 6.5
2	4	2,600	2,300	-11
3	4	3,200	3,120	- 2.5
4	5	750	560	-25.3
5	5	4,975	4,625	- 7
Avg				-10.4 ± 3.1
6	6	3,400	2,800	-17
7	6	2,620	2,370	- 9
"				-13 ± 4
8	7	3,470	3,170	- 8
9	7	3,080	2,435	-19
10	7	4,620	3,550	-23
"				-16.6 ± 4.46
11	8	4,690	3,980	-14
12	8	2,700	2,200	-18
13	8	2,910	2,350	-19.2
14	8	3,060	2,570	-16
"				-16.8 ± 0.37
15	9	4,320	3,600	-15
16	9	1,380	1,130	-18.1
17	9	2,080	1,770	-14.9
"				-16 ± 1
18	10	3,750	2,520	-32
19	10	2,970	2,570	-13
20	10	880	810	- 8
21	10	3,600	2,750	-23
22	11	3,700	2,550	-31
23	11	3,190	2,200	-31
24	11	4,190	3,280	-21
"				-22.7 ± 3.58
25	13	2,220	1,330	-40
26	13	4,310	4,070	- 5.5
27	14	3,100	1,780	-43
28	14	4,500	2,340	-48
"				-34.1 ± 9.55
29	16	4,600	3,800	-17
30	17	2,640	1,470	-44
"				-30.5 ± 13.52

N—Normal
A—Arthritis

several controls with muscles desiccated in the oven, at 105°C for 24 hours, to eliminate the excess of water

Results A Weight of Muscles in Arthritis

The results of the 30 experiments in this series given in Tables I and II, show that the percentage loss of weight of the tibialis anticus and the soleus is similar and varies with the

Changes in Weight of Muscles of Arthritic and Immobilized Arthritic Joints^{*}

P THOMSEN, J V LUCO, AND J E FRUGONE

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We have previously studied the synaptic mechanism in muscles of inflamed joints (Frugone, Thomsen, and Luco¹). In view of the results reported by Thomsen and Luco,² we thought it would be interesting to study the changes in weight of such muscles, and the possibility of modifying the weight through immobilization in different positions.

Methods Cats were used. Immobilization was carried out under ether or sodium pentobarbital (Nembutal Abbott) anesthesia, using bandages impregnated with plaster directly on the skin, extending from the metatarsal region

to just below the knee. The arthritis was produced by injecting 0.1 cc of a 5-10% solution of silver nitrate into the articular cavity of the tibia-tarsal joint. The cast was applied immediately after the injection of the silver nitrate. We immobilized in the extreme positions of extension and flexion, that is, in positions of forced hyperextension and hyperflexion held by the cast. The soleus and tibialis anticus muscles were weighed 2.5 to 16 days later, immediately after extirpation. As some of the animals developed marked edema of the immobilized extremity, we ran

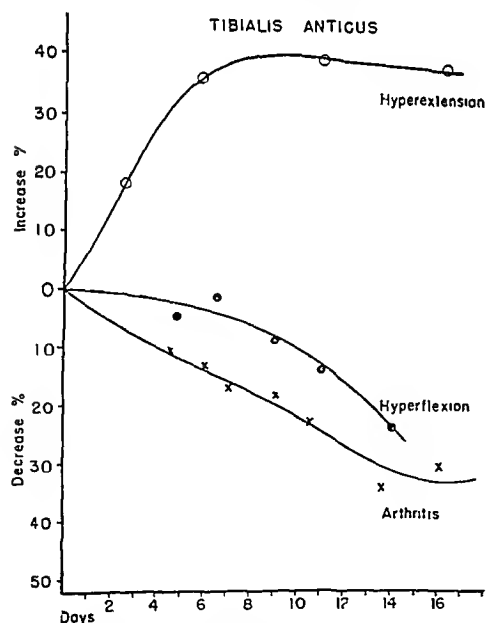


FIG 1

Changes of weight of the tibialis anticus in arthritis, arthritis with immobilization in hyperflexion, and arthritis with immobilization in hyperextension. The curves represent the plotted average values of Tables I, III, and V respectively.

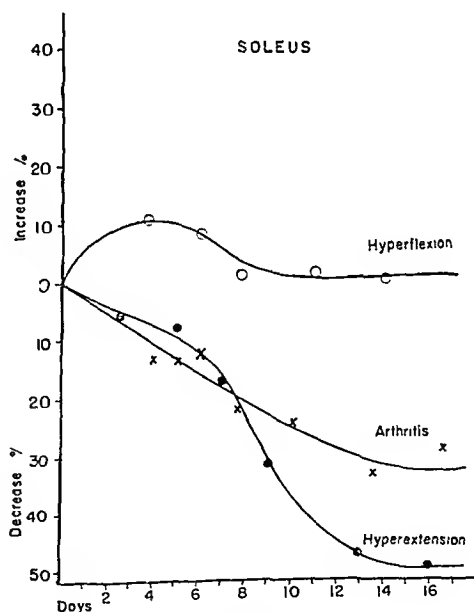


FIG 2

Changes of weight of the soleus in arthritis, arthritis with immobilization in hyperflexion, and arthritis with immobilization in hyperextension. The curves represent the plotted average values of Tables II, IV, and VI respectively.

^{*} Aided by a grant from the National Foundation for Infantile Paralysis.

¹ Frugone, J. E., Thomsen, P., and Luco, J. V.,

in press.

² Thomsen, P., and Luco, J. V., *J. Neurophysiol.*, 1944, 7, 245.

TABLE III
Tibialis in Arthritis with Hyperflexion

Animal No	Days of arthritis	Wt in g		% difference	Wt of desic muscles		% difference
		N	AI		N	AI	
61	2 5	1,950	1,890	— 3			
62	2 5	3,440	3,460	+ 0 5	940	890	— 5 3
63	2 5	2,100	2,150	+ 2 3	560	560	0
64	2 5	2,470	2,480	+ 0 4	685	700	— 2 1
65	2 5	1,150	1,080	— 6	350	325	— 7 1
	Avg 2 5			— 1 1 ± 1 15			
66	4	3,470	3,370	— 2 8			
67	5	3,410	3,320	— 2 6			
68	5	3,290	3,310	+ 0 6	860	850	— 1 1
69	5	4,970	4,320	— 13 1			
70	5	4,460	4,000	— 10 1			
71	5	3,300	3,300	0			
	" 4 8			— 4 6 ± 2 22			
72	6	4,820	4,930	+ 2 7			
73	6	2,270	2,080	— 8 4			
74	6	5,470	4,760	— 14 8	1,150	1,120	— 2 6
75	6	4,120	4,280	+ 3 8	1,130	1,160	+ 2 6
76	6	5,550	5,570	+ 0 3	1,430	1,420	— 0 7
77	7	3,370	2,870	— 15			
78	7	2,376	2,570	+ 11 9			
79	7	2,030	2,210	+ 8 7			
80	8	3,530	3,550	+ 0 5	1,040	940	— 9 6
	" 6 5			— 1 3 ± 3			
81	9	6,000	5,600	— 6 6			
82	9	1,970	1,780	— 10			
	" 9			— 8 3 ± 1 58			
83	11	3,350	3,600	+ 6 9			
84	11	3,870	2,840	— 26 8			
85	11	3,900	3,000	— 23			
86	11	2,950	2,640	— 10	700	610	— 12 8
	" 11			— 13 7 ± 7 61			
87	14	3,660	3,200	— 12 2	990	770	— 22 2
88	14	3,520	2,170	— 35 3	950	586	— 49 9
	" 14			— 23 7 ± 11 6			

N—Normal

AI—Arthritis with immobilization in hyperflexion

Immobilized in Hyperextension The results in the 30 animals studied are shown in Tables V and VI, and Fig 1 and 2. The soleus undergoes more atrophy than in the case of pure arthritis. The tibialis anticus shows an increase in weight that is evident both in the fresh and desiccated muscles. The figures for desiccated muscles are lower but tend to parallel those for fresh muscles.

Discussion The loss of weight caused in

the soleus and the tibialis anticus by an arthritis of the tibio-tarsal joint is very similar to that described by Lippman and Selig³. The curve obtained by those authors in a series of 40 animals, by plotting the loss of weight against the duration (in days) of arthritis, practically coincides with the curve that we

³Lippman, R. K., and Selig, S., *Surg Gyn Obst*, 1928, 47, 512

TABLE II
Showing Loss of Weight in Soleus Muscle in Arthritis

Animal No	Days of arthritis	Wt in g		% difference
		N	A	
1	4	2,970	1,800	-10
2	4	1,450	1,200	-17
3	4	1,850	1,620	-12
	Avg 4			-13 \pm 2
4	5	670	560	-16.4
5	5	2,375	2,120	-10
	" 5			-13.2 \pm 3
6	6	2,100	1,900	-9.5
7	6	2,240	1,900	-15
	" 6			-12.3 \pm 2.45
8	7	1,885	1,875	-5.5
9	7	1,970	1,520	-22
10	7	2,480	1,820	-26
11	8	2,550	2,200	-13
12	8	2,000	1,800	-10
13	8	2,680	1,520	-43.2
14	8	1,730	1,120	-35.2
	" 7.6			-21.4 \pm 5.55
15	9	2,300	1,930	-16
16	9	1,040	680	-34.6
17	9	1,510	920	-39
18	10	1,610	1,190	-26
19	10	2,580	2,050	-21
20	10	700	510	-27
21	10	1,900	1,700	-11
22	11	1,950	1,600	-19
23	11	2,240	1,530	-32
24	11	2,170	1,930	-15
	" 10			-24 \pm 2.91
25	13	1,350	1,050	-22
26	13	2,720	2,010	-26.1
27	14	1,880	1,160	-38
28	14	1,820	1,320	-27.4
	" 13.5			-33.3 \pm 4.35
29	16	2,930	2,420	-17
30	17	2,070	1,300	-37
	" 16.5			-27 \pm 10

N—Normal
A—Arthritis

number of days of arthritis. This relationship is seen more clearly in Fig. 1 and 2.

B Weight of Muscles of Arthritic Joints Immobilized in Hyperflexion. In this series we used 28 animals and the results are shown in Tables III and IV, and Figs. 1 and 2. In this position, the soleus after an initial increase

tends to maintain its normal weight. The tibialis anticus undergoes atrophy, but not as much as in the case of pure arthritis, although the weight of desiccated muscles shows that the loss of weight is similar to that produced by the arthritis alone.

C Weight of Muscles of Arthritic Joints

TABLE V
Tibialis in Arthritis with Hyperextension

Animal No	Days of arthritis	Wt in g		% difference	Wt of desic muscles		% difference
		N	AI		N	AI	
31	25	3,600	4,220	+17.2	1,080	1,170	+8.3
32	25	2,630	3,600	+36.8	780	870	+11.5
33	25	3,280	3,440	+4.8	920	980	+6.5
34	25	3,300	3,920	+18.8	820	1,020	+24.3
35	25	1,710	2,040	+19.4			
Avg 25				+18.2 ± 5.2			
36	5	5,750	6,500	+13			
37	5	3,020	3,990	+32.1			
38	5	2,900	3,960	+40			
39	5	3,170	4,640	+46.3	770	1,100	+42.8
40	5	4,480	7,270	+62.2	1,130	1,410	+24.7
41	5	3,560	5,000	+40.4	870	1,050	+17.1
42	7	4,500	6,220	+28			
43	7	3,280	4,320	+24			
44	7	3,180	4,380	+37.7	830	1,050	+26.5
" 57				+35.9 ± 4.68			
45	9	5,200	6,250	+17			
46	9	4,280	5,560	+22.8	1,110	1,220	+9.9
47	9	3,250	3,820	+17.5	830	960	+15.5
48	9	3,420	4,610	+25.8	840	1,120	+33.3
49	9	2,250	3,130	+39.1			
50	10	3,940	5,300	+26			
51	12	3,780	5,460	+17.9	1,020	1,220	+19.6
52	13	2,360	4,420	+87.2	590	970	+64.4
53	13	4,200	4,040	-3.8			
54	13	1,330	2,610	+96.2	380	640	+68.4
55	14	1,510	2,760	+82.8	440	700	+59
" 108				+38.9 ± 10			
56	16	3,900	5,600	+43.6	1,050	1,390	+32.3
57	16	2,830	3,920	+38.5	790	1,060	+34.1
58	16	2,720	3,170	+15.2			
59	16	3,640	5,980	+64.5	1,000	1,190	+19
60	16	4,610	6,430	+25.3	1,280	1,620	+26.7
" 16				+37.2 ± 8.6			

N—Normal

AI—Arthritis with immobilization in hyperextension

the same as that with hyperextension alone

Hyperflexion results in a slight increase in weight of the soleus, and arthritis plus hyperflexion results in no change of weight the hyperflexion preventing the loss of weight that usually accompanies arthritis

In the case of the tibialis anticus, in which arthritis causes a loss of and hyperextension an increase of weight, the simultaneous action of these two factors results in an increase of weight greater than by immobilization alone. No doubt edema plays a role in these results. Also, the standard error of the increase in weight is ± 10 , indicating that the mean value

may be subject to great variation

Hyperflexion causes a loss of weight of the tibialis, but when combined with arthritis it fails to alter the loss of weight due to the latter

Summarizing the loss of weight produced by immobilization and shortening of a muscle is not modified by arthritis in a period of 10 or 11 days. On the other hand, the atrophy due to arthritis does not develop, or is changed into hypertrophy when the joint is immobilized in a position which by itself causes an increase in weight

As we said before, the increase in weight of the tibialis by immobilization and arthritis

TABLE IV
Soleus in Arthritis with Hyperflexion

Animal No	Days of arthritis	Wt in g		% difference	Wt of diseased muscles		% difference
		N	AI		N	AI	
61	2 5	1,210	1,530	+26.2			
62	2 5	1,730	1,950	+11.2	450	480	+ 6.2
63	2 5	1,180	1,220	+ 3.4	310	330	+ 6.4
64	2 5	1,060	1,220	+15	300	345	+15
65	2 5	750	1,000	+31.8	220	280	+27.2
66	4	2,440	2,340	- 4.1			
67	5	2,370	2,460	+ 3.6			
68	5	2,430	2,360	- 2.8	540	560	+ 3.7
69	5	2,200	2,770	+25.8			
70	5	2,620	2,740	+ 4.6			
71	5	1,830	1,890	+ 3.2			
	Avg 3 7			+10.7 ± 3.74			
72	6	2,700	3,000	+11			
73	6	1,330	1,470	+10.5			
74	6	2,250	2,380	+14.6	520	600	+15.3
75	6	3,000	2,930	- 2.3	770	770	0
76	6	2,480	2,690	+ 8.4	630	640	+ 1.6
	" 6			+ 8.4 ± 2.82			
77	7	2,030	1,450	-28.5			
78	7	1,050	1,080	+ 2.8			
79	7	1,530	2,018	+32			
80	8	2,670	2,400	-10.1	670	610	- 8.9
81	9	3,650	3,450	- 5.4			
82	9	1,370	1,620	+18.3			
	" 7 8			+ 14.5 ± 8.78			
83	11	1,850	2,220	+15.8			
84	11	2,220	2,370	+ 6.4			
85	11	2,550	2,410	- 5.5			
86	11	1,790	1,400	-11.9	380	360	- 5.2
	" 11			+ 1.7 ± 5.66			
87	14	1,870	1,850	- 1	450	450	0
88	14	2,550	2,600	+ 1.9	630	650	+ 3.1
	" 14			+ 0.4 ± 1.58			

N—Normal

AI—Arthritis with immobilization in hyperflexion

obtained for the tibialis in a series of 30 animals

The muscular atrophy produced by arthritis is different from that obtained by immobilization and as observed by Thomsen and Luco, in the former case both muscles undergo atrophy, while in the latter case only the muscle that is in a state of maximum shortening undergoes atrophy

If we combine these two conditions that cause changes of weight, *i.e.*, arthritis and immobilization in an extreme position, we

observe that in some cases we obtain a summation while in others we do not. In Table VII, we show the average percentage of change in weight in the group of animals studied after 10-11 days of treatment, together with the results obtained by Thomsen and Luco² for the same period.

Table VII shows that the loss of weight of the soleus in arthritis is slightly over one-half the loss of weight in immobilization in hyperextension, and that the loss of weight in arthritis with hyperextension is practically

TABLE VII

	% diff in soleus	% diff in tibialis
Arthritis	-24.0	-22.7
Arthritis and hyperextension	-38.8	+38.9
Hyperextension	-38.0	+28.0
Arthritis and hyperflexion	+2.9	-11.6
Hyperflexion	+11.0	-13.0

of maximum extension (hyperextension) caused a greater loss of weight than immobilization in maximum shortening, and that the change in the soleus was the same in either position. The experimental conditions were different in the two cases, and we believe that the different results are due to the fact that we studied the first 16 days of immobilization while Huddleston's experiments extended from 95 to 290 days. It may be that our results would change with time and come to resemble those of Huddleston. Besides, the results listed in our tables show that there are wide variations from animal to animal and, therefore, the averages must be taken from a large number of animals.

Our results cannot be compared either with those of Hines⁵ who studied in rats the effect of immobilization on the recovery from

⁵ Hines, H. M., *J. A. M. A.* 1942, 120, 515

paralysis caused by crushing the nerve. In our experiments the nerve was kept intact.

Summary 1 Arthritis of the tibio-tarsal joint produced in cats by injecting silver nitrate, causes an atrophy of the soleus and tibialis anticus muscles. Tables I and II, Fig 1 and 2.

2 Immobilization of the inflamed tibio-tarsal joint in hyperflexion results in a loss of weight of the tibialis that is similar or slightly less marked than that caused by arthritis alone. Table III, Fig 1. In these conditions the soleus keeps its normal weight. Table IV, Fig 2.

3 Immobilization of the inflamed joint in hyperextension results in an increase of weight of the tibialis. Table V, Fig 1. The soleus, on the other hand, shows a greater loss of weight than by arthritis alone. Table VI, Fig 2.

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Respiratory Mask and Valves for Dogs

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The units to be described were developed in this laboratory for the purpose of collecting expired air of unanesthetized, trained dogs. The avoidance of leaks, minimization of resistance to breathing and of dead space were prime factors considered in designing the apparatus. No new fundamental principles are involved, rather, the apparatus embodies

good features of older designs, eliminates some of the bad and utilizes new devices, to our knowledge never previously described.

Mask The mask consists of a copper wire frame, on the order of carefully shaped commercial wire muzzle made to fit loosely the snout of a 15 kg mongrel hound. The nasal end is projected about 1" beyond the nose and fitted with a No. 9 stopper with a single $\frac{3}{8}$ " bore. The open end is bent to fit the snout near the lip corners with about $\frac{3}{4}$ "

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TABLE VI
Soleus in Arthritis with Hyperextension

Animal No	Days of arthritis	Wt in g		% difference	Wt of desicc muscles		% difference
		N	AI		N	AI	
31	2 5	2,080	1,850	-11.1	540	510	-5.5
32	2 5	1,730	1,740	+0.5	450	450	0
33	2 5	1,250	1,360	+8.8	360	380	+5.5
34	2 5	2,330	2,070	-11.1	630	560	-11.1
35	2 5	1,370	1,200	-12.2			
	Avg 2 5			-5.4 ± 4.32			
36	5	2,850	2,800	-2			
37	5	1,740	1,630	-6.3			
38	5	1,950	1,710	-12			
39	5	1,800	1,230	-31.6	420	320	-23.8
40	5	1,740	1,680	-3.4	440	390	-11.3
41	5	1,480	1,640	-10.6	420	430	+2.3
	" 5			-7.7 ± 5.63			
42	7	3,100	3,000	-3			
43	7	1,600	1,400	-12			
44	7	2,300	1,490	-35.2	520	370	-28.9
	" 7			-16.7 ± 9.5			
45	9	2,550	1,830	-28			
46	9	2,080	1,350	-35.5	450	330	-26.6
47	9	1,700	890	-47.6	470	290	-38.3
48	9	1,950	1,340	-31.2	470	310	-34
49	9	1,320	1,040	-21.2			
50	10	1,970	1,430	-27			
	" 9 1			-31.6 ± 3.16			
51	12	1,690	1,130	-33.1	460	280	-39.1
52	13	1,740	900	-48.2	440	220	-50
53	13	1,820	940	-48.4			
54	13	1,060	440	-58.5	275	110	-60
55	13	1,160	600	-48.3	310	170	-45.1
	" 12 8			-47.3 ± 4.15			
56	16	2,640	1,330	-45.4	670	330	-50.7
57	16	1,730	660	-62	430	190	-55.8
58	16	1,060	610	-69			
59	16	2,090	1,510	-27	550	290	-47.2
60	16	2,270	1,290	-43.7	620	360	-41.8
	" 16			-49.4 ± 7.36			

N—Normal

AI—Arthritis with immobilization in hyperextension

is less marked in desiccated muscles. This applies to both positions. On the other hand, the results obtained with desiccated soleus muscle are similar to those obtained with the fresh muscle.

With reference to peri-arthritis, Lippman and Selig discarded all animals that developed this complication and, nevertheless, ob-

tained a curve that almost coincides with the one we obtained without discarding such animals.

The results obtained in this work do not agree with those of Huddleston,⁴ who found that immobilization of the tibialis in a position

⁴ Huddleston, O. L., *South. M. J.*, 1944, **37**, 72

close to the vertical position as shown in the diagram as otherwise the flaps will be opened by gravity between respirations allowing significant leaks. Small springs to close the flaps might eliminate this danger. The valve should be stored in the vertical position when not in use. The tube (B) with a 45° bend, inserted into the stopper of the mask, is used when the

dog is supine with head tilted 45° upward. Modification of this bend permits use with the animal in any desired position. Modification of the diameter and bend of (B) and attachment to a suitable mask permits use on man. No significant resistance to respiration develops with these valves even during exercise or CO₂ inhalation.

15221

Effect of Salicylate on Plasma Fibrinogen and Sedimentation Rate in Rheumatic and Non-Rheumatic Patients *

S. RAPOPORT AND GEORGE M. GUEST

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Despite the long-standing use of salicylates in the management of rheumatic fever, considerable uncertainty still exists with regard to their efficacy and to their mode of action in this disease. While some observers believe that their clinical effect may be ascribed entirely to the analgesic and antipyretic action,¹ others have assumed a specific inflammation-suppressant effect of these drugs in rheumatic fever. Studies which indicated a more rapid return to normal of the sedimentation rate in rheumatic patients receiving large doses of salicylates were interpreted as supporting evidence for this view, under the assumption that the sedimentation rate represented an index of inflammatory activity.² A specific influence of the drug on the sedimentation rate apart from its assumed action on the inflammation is also suggested by the observation that the sedimentation rate may increase promptly on discontinuance of salicylate therapy.³

Present evidence indicates that the sedimentation rate of the erythrocytes is mainly

dependent on the concentration and distribution of the various fractions of the plasma proteins.⁴ Fibrinogen exerts the greatest accelerating action. A close relationship between the sedimentation rate and the level of fibrinogen has been demonstrated by several observers in various experimental conditions and diseases, among them rheumatic fever.⁵ Fibrinogen is generally believed to be formed solely in the liver.⁶ In view of these facts it appears possible that the effect of salicylates on the sedimentation rate of rheumatic patients is due to an inhibition of the formation of fibrinogen by the liver. That salicylates may affect liver function is also suggested by the observation that they may cause significant reduction in the plasma prothrombin in animals and in man.⁷ One would not expect such

* Reported at a conference on Liver Injury held under auspices of the Josiah Macy, Jr. Foundation, New York, September 19, 1944.

¹ Hamlik, P. I., *Medicine*, 1926, **5**, 197.

² Coburn, A. F., *Bull. Johns Hopkins Hosp.*, 1943, **73**, 435.

³ Lichty, J. A., and Hooker, S. P., *Proc. Soc. Exp. Biol. and Med.*, 1941, **48**, 69.

⁴ Fahreus, R., *Physiol. Rev.*, 1929, **9**, 241; Westergren, A., Theorell, H., and Widstrom, S., *Gen. Exp. Med.*, 1931, **75**, 668; Bendien, W. M., Neuberg, I., and Snapper, I., *Bioch. Z.*, 1932, **247**, 106; Gies, S. J., and Mitchell, E. B., *Proc. Soc. Exp. Biol. and Med.*, 1942, **51**, 403.

⁵ Ernestine, A. C., *Am. J. Med. Sc.*, 1930, **180**, 12; Gilligan, D. R., and Ernestine, A. C., *Am. J. Med. Sc.*, 1934, **187**, 552; Ham, T. H., and Curtis, P. C., *Medicine*, 1938, **17**, 447.

⁶ Midden, S. C., and Whipple, G. H., *Physiol. Rev.*, 1940, **20**, 194.

⁷ Link, K. P., Overman, R. S., Sullivan, W. R., Huebner, C. F., and Scheel, L. D., *J. Biol. Chem.*

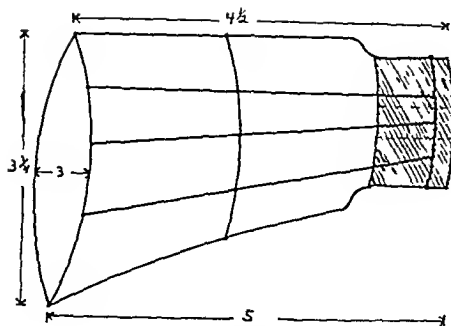


FIG 1

Mask frame without covering Shaded portion is No 9 rubber stopper for insertion of B in Fig 2

clearance Eight longitudinal wires spaced evenly around the frame serve to give good support and shape to the covering material The finished frame (Fig 1) is wrapped with 3 or 4 thicknesses of plaster bandage, allowed to dry overnight and soaked for an hour in melted paraffin A latex surgeon's glove, size 8, from which the fingers are removed is stretched over the open end of the mask and secured by wrapping tightly with string and brushing with molten paraffin about 3" of the wrist end of the glove is allowed to project as a sleeve In use, the sleeve is stretched over the dog's muzzle to beyond the lip corners and invaginated into the mask by sliding the latter toward the eyes Two rubber strips cemented to the end of the sleeve and tied together behind the ears prevent rolling of the glove and exposure of the lip corners during a measurement We have found no grease to be necessary to prevent leaks under the glove, this may, however, be necessary in the case of very long-haired animals The mask can be steadied during the experiment either by hand or a clamp Such a mask will fit a number of 10-20 kg animals with similar muzzle shapes thus obviating the necessity of individually molded outfits For smaller animals a smaller size glove may be used as sleeve, but the mask dead-space factor may become significant in such cases Hence we have constructed similar but smaller size masks which may be used for 5-10 kg dogs Although this mask does not fit the muzzle tightly enough to prevent some opening of the jaws it would limit the extent of such movement and thus

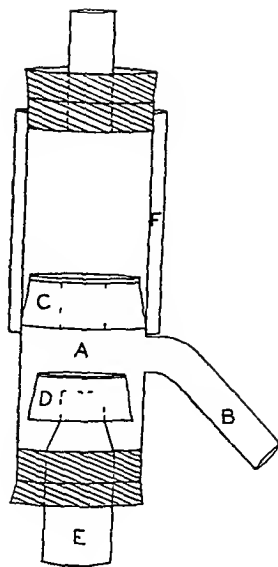


FIG 2

Respiratory valves, shown in position used

might limit the efficiency and degree of the panting process

Valves The valves are of the ordinary rubber flap type enclosed in glass (A) in Fig 2 is a 5 1/4" long, 45 mm o d standard Pyrex tube with a 13 mm o d tube (B) fused to one side about 1/3 of the distance from one end The expiratory valve (C) is a No 8 rubber stopper with 5/8" bore covered with a circle of 1/32" rubber sheet secured to the stopper by a small dot of rubber cement on one edge A perfectly flat piece of sheet rubber and a smooth stopper must be selected to avoid leaks The inspiratory valve (D) is a No 7 stopper covered with a similar flap The 25 mm o d glass tube (E) should produce a minimum of stretching of the stopper (D) when inserted to prevent distortion of the valve seat and consequent leaks The ends of (A) are closed with single bore No 9 stoppers which can be connected to spirometers A 20-watt electric heater (F) is built to fit over the upper half of the valve This prevents condensation of moisture on the expiratory valve during collection and eliminates the objectionable sticking of this flap with its consequent resistance to expiration The valve should be used only in or very

tration in patients suffering from various chronic disorders in which the sedimentation rate is elevated

Material and Methods Fifteen patients with elevated sedimentation rates, varying in age from 2 to 14 years, were chosen for this study. Eight displayed some manifestations of rheumatic fever, 4 were suffering from lipoid nephrosis, and 3 from chronic osteomyelitis. The subjects received daily 0.09-0.22 g per kg of body weight of acetyl-salicylic acid combined with an equal amount of sodium bicarbonate for a period of 7 to 29 days. Blood samples for the determination of the sedimentation rate and of the plasma fibrinogen were obtained before, during, and after the period of medication at varying intervals of time, usually 3 to 5 days. Most subjects tolerated the drug well, except for one patient with nephrosis who developed hypoprothrombinemia and hematuria, shortly after discontinuance of salicylate medication the hematuria ceased and the prothrombin returned to normal.

The sedimentation rate was determined by the method of Rourke and Ernstene⁸ using heparinized blood with values corrected for the packed volume of cells. Fibrinogen was determined as fibrin, following essentially the procedure of Cullen and Van Slyke.⁹

Results Ten of the patients, whose data are summarized in Table I, exhibited significant reduction in the fibrinogen level of their plasma during periods of salicylate medication.

⁸ Rourke, M. D., and Ernstene, A. C., *J. Clin. Invest.*, 1930, **8**, 545.

⁹ Cullen, G. E., and Van Slyke, D. D., *J. Biol. Chem.*, 1920, **41**, 587.

In all instances the sedimentation rate of the erythrocytes was also reduced, but the extent of the decrease varied considerably. In the other 5 patients, in whom the plasma fibrinogen did not decrease, no important changes were observed in the sedimentation rate. Several striking discrepancies between the two indices, demonstrated particularly in Cases 3, 5, and 8 in Table I, would indicate that other factors besides fibrinogen were operative in determining the sedimentation rate. It well may be that the effect of the salicylates on the distribution of plasma protein, and with it on the sedimentation rate, is not limited to fibrinogen alone, although determination of albumin and globulin by the conventional Howe method in several of the samples did not indicate significant change. The data on Cases 1 to 6 would indicate that salicylates may lower the plasma fibrinogen and the sedimentation rate in conditions unrelated to the rheumatic disorder. Such findings justify the assumption that salicylates exert an unspecific action on the liver, and indirectly support the contention of those observers who believe that salicylates do not have a specific anti-rheumatic action. However, it is of course conceivable that changes in the distribution of plasma protein produced by salicylates may in some manner influence favorably the course of rheumatic inflammation.

Summary In 10 of 15 patients with elevated sedimentation rates, suffering from non-rheumatic and rheumatic disorders, the sedimentation rate and concentration of fibrinogen decreased following salicylate therapy. It is suggested that the change in the fibrinogen may represent an effect of salicylate on the liver.

TABLE I
Changes in Plasma Fibrinogen and Sedimentation Rate Following Salicylate Therapy

Case No	Diagnosis	Age, yrs	Dosage, g/kg/day	Time relative to medication	Plasma fibrinogen, mg/100 cc	Sedimentation rate	
						uncorrected, mm/min	corrected, mm/min
1	Nephrosis	11	0	before	951	3.63	1.96
			0.13	2 days	857	3.07	1.80
			0.13	5 "	676	2.86	1.64
			0.13	10 "	534	2.35	1.33
			0.13	14 "	468	2.26	1.42
			0	8 " after	735	2.65	1.90
2	"	7	0	20 " "	764	3.09	2.00
			0	before	924	3.72	2.18
			0.15	16 days	652	3.00	1.88
3	"	8	0	8 " after	794	4.22	2.14
			0	before	653	2.20	2.00
			0.11	9 days	415	1.70	1.38
			0.11	16 "	184	1.27	1.02
4	Chion staphylococcal osteomyelitis	6	0	7 " after	479	1.55	1.33
			0	before	628	2.81	1.68
			0.09	7 days	360	2.52	1.48
			0	7 " after	690	3.15	1.83
5	" " "	3	0	before	501	1.64	1.04
			0.11	7 days	220	1.06	0.65
			0	14 " after	598	1.06	0.68
6	Brain abscess	14	0	before	858	4.64	1.77
			0.15	4 days	780	2.49	1.40
			0.15	13 "	583	1.24	1.18
7	Rheum fever	2	0	before	843	2.50	1.35
			0.20	7 days	608	1.34	0.72
			0.20	20 "	376	0.73	0.45
8	" "	12	0	before	858	2.71	1.60
			0.13	5 days	589	2.26	1.42
			0.13	20 "	404	0.52	0.28
9	" "	5	0	45 " after	631	0.97	0.78
			0	before	622	1.86	1.18
			0.17	7 days	552	0.80	0.36
10	" "	7	0.17	29 "	388	0.19	0.12
			0	before	614	2.83	1.74
			0.13	14 days	548	2.19	1.18
11	Nephrosis	7	0	12 " after	673	2.32	1.50
			0	before	785	6.50	2.10
			0.14	7 days	808	6.74	2.12
12	Brain abscess	8	0	7 " after	940	7.57	2.57
			0.13	before	726	1.16	0.68
13	Rheumatoid arthritis	11	0	12 days	746	1.34	0.74
			0	before	626	2.41	1.70
			0	10 days	640	2.81	1.94
14	Pericarditis und etiol	2	0	12 " after	740	4.22	2.44
			0	before	472	1.26	0.72
			0.18	6 days	506	1.16	0.63
15	Rheum fever	14	0	before	619	—	—
			0.13	9 days	592	0.80	0.66
			0.13	16 "	614	1.13	0.80

an effect of the salicylates to be limited to rheumatic patients alone, decreases in the

1943, 147, 463, Rappoport, S, Wing, M, and Guest, G M, PROC SOC EXP BIOL AND MED, 1943, 53, 40, Meyer, O O, and Howard, B, PROC SOC EXP BIOL AND MED, 1943, 53, 234, Shapiro, S, Redish, M H, and Campbell, H A, PROC SOC EXP BIOL AND MED, 1943, 53, 251

plasma fibrinogen and in the sedimentation rate following salicylate medication should be demonstrable in other patients with elevated sedimentation rates, who do not suffer from rheumatic fever. In the present study parallel determinations of the sedimentation rate and of plasma fibrinogen were carried out before, during, and after courses of salicylate adminis-

TABLE I
Survival of Penicillin sensitive *Staphylococcus* in Agar Containing Penicillin

Survival in agar by colony counts			
Penicillin, units/ml	Strain NRRL bacteria/ml	Strain 62 bacteria/ml	Strain 3890 bacteria/ml
0.01	2.2×10^8	2.4×10^8	2.7×10^8
0.02	2.5×10^8	2.5×10^8	2.0×10^8
0.05	1.0×10^8	2.5×10^8	9.0×10^7
0.1	2.0×10^8	7×10^7	3.0×10^7
0.2	1.5×10^8	6×10^7	
0.5	1.0×10^7	"	1.0×10^7
1.0	0	0	0

TABLE II
Survival of *Staphylococcus* Made Resistant to Penicillin

		Penicillin, units/ml	Survival by colony count bacteria/ml
a	Strain from a colony grown in presence of	0.05 units/ml	
		0.01	7×10^8
		0.02	2×10^8
		0.05	7×10^7
		0.1	2×10^8
		0.2	4×10^7
		0.5	2×10^7
		1.0	0
b	Strain	0.2	
		0.0	7×10^8
		0.2	9×10^7
		0.5	2×10^7
		1.0	1×10^7
		2.0	2×10^6
		5.0	2×10^5
		10.0	0
c	Strain	1.0	
		0.0	7×10^8
		1.0	2×10^8
		2.0	2×10^7
		5.0	1×10^7
		10.0	2×10^6
		50.0	1×10^5
		120.0	0

stock solutions of 10,000 units per ml in phosphate buffer of pH 6.9. The stock was titrated at frequent intervals by the cup method⁷ and compared with a penicillin standard. A stock was discarded as soon as its titer began to diminish appreciably.

Tests for penicillinase activity were made by mixing 1 ml of the sample to be tested with 1 ml of a penicillin solution of the desired concentration (generally 40 units/ml). The mixtures were incubated at 37°C for 1 or 2 hours then assayed directly or after dilution by the cup method. Acetone-ether

dried preparations for penicillinase tests were made according to Harper.⁸

Results When sensitive strains of staphylococci are plated in agar containing penicillin the results are of the type illustrated in Table I.

If bacteria from any of the colonies grown in the presence of the largest amount of penicillin in the experiments of Table I are picked out, grown in broth, and retested in penicillin agar, results of the type illustrated in Table II a are obtained. By sampling out and testing colonies grown in the presence of progressively increasing amounts of

⁷ Sand, W. H., and Morse, A. J., *J. Bact.* 1944 47, 190.

⁸ Harper, G. L., *Lab.* 1945 245 569.

A Test for Penicillin Sensitivity and Resistance in *Staphylococcus* *

S E LURIA † (Introduced by E C MacDowell)

From the Department of Genetics, Carnegie Institution of Washington, Cold Spring Harbor, N Y

The occurrence of penicillin-fast strains of bacterial species generally susceptible to the action of penicillin has been recorded repeatedly¹. It has been established that a susceptible strain may acquire penicillin resistance *in vivo* following penicillin treatment, or *in vitro* after growth in penicillin-containing media. The mechanism of acquisition of penicillin fastness *in vitro* by staphylococci was studied by Demerec² who found that even very susceptible strains of staphylococci give origin to a few variants resistant to small concentrations of penicillin. The variants in turn, once allowed to grow, give variants of higher resistance. In several steps, but never in one step, it is possible to obtain variants resistant to very high concentrations, up to 250 or more units per ml. This type of resistance is generally permanent. Using the statistical method devised by Luria and Delbruck³ for the study of bacteriophage resistance, Demerec was able to show that these penicillin-fast variants arise by rare mutations from sensitive organisms, independently of the action of penicillin itself. In all probability the drug

merely acts as a selective agent which, by inhibiting the growth of sensitive individuals, allows the mutant individuals of sufficient resistance to grow. Resistance to high concentrations is the result of successive mutations, each contributing further resistance.

The situation is complicated by the occurrence of another type of penicillin resistance in staphylococci¹. Many of the so-called resistant strains isolated from patients, either before or after treatment with penicillin, seem to owe their ability to withstand the antibiotic to the production of a penicillin inactivator, probably of enzymatic nature (penicillinase⁴). Experiments discussed in this paper show that penicillinase production need not be associated with penicillin resistance of the individual cells.

One important requirement in experimental work and in hospital laboratory routine is the availability of a reliable test for penicillin resistance in staphylococcus. The test should indicate the degree of sensitivity of the bulk of a bacterial population and the presence and proportion in it of cells of higher resistance. For penicillinase-producing strains, the test should indicate the degree of sensitivity of individual cells. Such a test is described in the present paper.

Technic For the experiments on survival in agar containing penicillin pour plates were used. These were made by introducing the desired amount of penicillin, and then a measured bacterial inoculum, into a tube containing 10 ml of melted nutrient agar (at 45°C), mixing the contents of the tube thoroughly, and plating immediately. With this method, no heat inactivation of penicillin takes place. Results were read after 72 hours of incubation at 37°C.

Penicillin was kept in a refrigerator in

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† The author is indebted to Lt. A. Reiss, Sn C, Base Hospital Laboratory, Mitchell Field, N Y, and to Miss B. Johnson, Surgical Bacteriology Laboratory, College of Physicians and Surgeons, Columbia University, for supplying cultures of staphylococci, and to Chas. Pfizer and Co. for generous supplies of penicillin. The technical assistance of Miss Rachel M. Arbogast is gratefully acknowledged.

¹ See Spink, W. W., Hall, W. H., and Ferris, V., *J. A. M. A.*, 1945, **128**, 555.

² Demerec, M., *Proc. Nat. Acad. Sc.*, 1945, **31**, 16.

³ Luria, S. E., and Delbruck, M., *Genetics*, 1943, **28**, 491.

⁴ Abraham, E. P., and Chaim, E., *Nature*, 1940, **146**, 837.

TABLE I
Survival of Penicillin sensitive *Staphylococcus* in Agar Containing Penicillin

Survival in agar by colony counts			
Penicillin, units/ml	Strain NRRL, bacteria/ml	Strain 02, bacteria/ml	Strain 5399, bacteria/ml
0.00	2.2×10^8	2.4×10^8	2.7×10^8
0.01	2.5×10^6		
0.02	2.5×10^4	5.0×10	2.0×10^4
0.03	1.0×10^4	4.5×10^2	9.0×10^2
0.04	4.0×10^3	5×10^1	3.0×10^1
0.06	1.3×10^2	6×10^0	
0.08	3.0×10^1	0	1.0×10^1
0.10	0	0	0

TABLE II
Survival of *Staphylococcus* Made Resistant to Penicillin

		Penicillin, units/ml	Survival by colony count, bacteria/ml
a	Strain from a colony grown in presence of	0.06 units/ml	
		0.00	5×10^8
		0.04	3×10^8
		0.08	5×10^5
		0.12	2×10^1
		0.16	4×10^1
		0.24	2×10^2
b	<i>idem</i>	0.22	0
		0.4 "	
		0.0	7×10^8
		0.2	9×10^6
		0.4	3×10^5
		0.4	1×10^5
		0.5	2×10^3
c	<i>idem</i>	0.75	2×10^2
		1.00	0
		1.0 "	
		0.0	6×10^8
		1.0	4×10^4
		2.0	1×10^1
		4.0	1×10^2
		6.0	2×10^1
		8.0	1×10^1
		12.0	0

stock solutions of 10,000 units per ml in phosphate buffer of pH 6.9. The stock was titrated at frequent intervals by the cup method,⁵ and compared with a penicillin standard. A stock was discarded as soon as its titer began to diminish appreciably.

Tests for penicillinase activity were made by mixing 1 ml of the sample to be tested with 1 ml of a penicillin solution of the desired concentration (generally 40 units/ml). The mixtures were incubated at 37°C for 1 or 2 hours, then assayed, directly or after dilution, by the cup method. Acetone-ether

dried preparations for penicillinase tests were made according to Harper.⁶

Results When sensitive strains of staphylococci are plated in agar containing penicillin, the results are of the type illustrated in Table I.

If bacteria from any of the colonies grown in the presence of the largest amount of penicillin in the experiments of Table I are picked out, grown in broth, and retested in penicillin agar, results of the type illustrated in Table II, a, are obtained. By sampling out and testing colonies grown in the presence of progressively increasing amounts of

⁵ Schmidt, W. H., and Moyer, A. J., *J. Bact.*, 1944, **47**, 199.

⁶ Harper, G. J., *Lancet*, 1943, **245**, 569.

TABLE III

Survival of Penicillinase producing Staphylococci in Agar Containing Penicillin

	Growth in penicillin agar		
	Penicillin Units/ml	Inoculum, per plate	
		5×10^7 Colony count*	5×10^5 Colony count*
a <i>Staphylococcus albus</i> Shaeffer	0.00	Continuous growth	5×10^5
	0.00	"	10^4
	1.00	"	2×10^3
	2.00	"	3×10^2
	5.00	"	0
	10.00	10^5	0
b <i>Staphylococcus albus</i> Cleaves	0.00	Continuous growth	5×10^5
	0.05	"	10^4
	0.20	"	1×10^3
	0.50	10^5	5×10^{-1}
	1.00	10^4	0
	2.00	1.9×10^1	0

* Continuous growth corresponds to more than 1×10^6 colonies per plate. Counts higher than 1×10^3 are estimates.

TABLE IV

Growth of Sensitive Staphylococci in Agar and Broth Containing Penicillin

Platings in agar			Inoculations in broth Inoculum, bacteria per tube									
Penicillin, units/ml	Survival by colony counts bacteria/ml	Survival ratio	2.8×10^7		2.8×10^6		2.8×10^5		2.8×10^4		2.8×10^3	
			(a)	(b)	(a)	(b)	(a)	(b)	(a)	(b)	(a)	(b)
0.00	2.8×10^8	1	+	+	+	+	+	+	+	+	+	+
0.01	1.0×10^6	3.5×10^{-2}	+	+	+	+	+	+	+	+	+	+
0.02	5.0×10^4	1.8×10^{-4}	+	+	+	+	+	+	—	—	—	—
0.04	2.0×10^2	7.1×10^{-7}	+	+	+	+	—	—	—	—	—	—
0.06	1.3×10^2	4.6×10^{-7}	+	+	±	+	—	—	—	—	—	—
0.08	3.0×10^1	1.1×10^{-7}	+	+	—	—	—	—	—	—	—	—
0.10	5×10^0	2×10^{-8}	—	—	—	—	—	—	—	—	—	—

(a) = predicted, (b) = experimental, + = visible growth, — = no visible growth

penicillin, results are finally obtained like those shown in Table II, b and c. The strain of Table II, c, appears to be already quite resistant. These results fully confirm those of Demerec.²

The data in Tables I and II were obtained by plating suitable numbers of bacteria in agar containing penicillin, in these cases, plates with the same concentration of penicillin and with different inocula give counts proportional to the size of the inoculum. When some of the "resistant" strains isolated from patients are tested, however, the results are often of the type illustrated in Table III. Here the survival, given by colony counts for each concentration of penicillin, is not proportional to the size of the

inoculum, but is much higher for heavy inocula.

Each one of the strains giving results of this latter type was found by proper tests to produce penicillinase. Penicillin inactivation was effected both by the whole bacterial cultures and by acetone-ether dried preparations according to Harper.

Results like those recorded in Table III show that the protection afforded by penicillinase is a mass phenomenon, occurring only when bacteria are inoculated in large number. The initial presence of many individuals is probably needed to yield protective amounts of penicillinase. The amounts of extracellular penicillinase carried over with a heavy inoculum are not responsible for the

protection, as proved by experiments in which the inoculum consisted of thrice washed cells the results were similar to those with the whole culture. Moreover, if one uses a small inoculum suspended in the clear centrifugate of a fully grown culture, the amount of survival is not appreciably higher.

It is worth mentioning, incidentally, that in our work we found very little penicillinase activity in the supernatant of penicillinase-producing cultures, and no activity was recovered by filtration through sintered glass, Seitz, or Mandler filters, not even after eluting the filters with phosphate buffer.

An important conclusion from experiments like those represented in Table III is that, when penicillin resistance is of the "penicillinase" type, the individual cells may be much more sensitive than the strain appears to be when tested as a whole. In cases like that illustrated by Table III, b, the individual organisms are almost as sensitive as the cells of a sensitive strain.

Platings in penicillin agar, like those described in Tables I-III, represent a good test for penicillin resistance. In practice, however, the method of quantitative platings is not convenient because of its technical complexity. It was therefore desirable to find out if corresponding results could be obtained in liquid medium.

For bacterial strains that do not produce penicillinase, we may expect that, if the bacteria are inoculated into liquid medium containing various concentrations of penicillin, visible growth will appear whenever the inoculum contains at least one individual resistant to the concentration of penicillin present. By using different inoculum-sizes, it should be possible to estimate the proportion of resistant individuals.

For bacterial strains that produce penicillinase, we may expect visible growth whenever enough penicillinase is produced to destroy the amount of penicillin present. If most cells of the strain are sensitive there should be a large discrepancy between tubes with large and with small inocula. In all cases, therefore, *important inoculum-size effects are to be expected*.

These expectations were fully confirmed

by experiments. Table IV shows the results of a typical experiment with a sensitive strain, in which different amounts of bacteria from the same culture were introduced into penicillin broth and into penicillin agar. From the colony counts in agar the expectation of growth in each tube was predicted (columns a) and compared with experimental results (columns b). Predicted and actual results coincide satisfactorily.

Similar results were obtained with all strains not producing penicillinase, whatever their degree of resistance. Visible growth in broth appears whenever the inoculum contains at least one individual resistant to the concentration of penicillin employed.

In similar tests for the strains producing penicillinase, the tubes with heavy inoculum showed growth even with very high concentrations of penicillin, the tubes with small inoculum did or did not show growth depending on the sensitivity of the individual cells, as indicated by independent measurements of survival in agar (Table III).

For the purpose of practical measurement of penicillin resistance, we recommend the following test, which can easily be performed in hospital laboratories and whose results can be read within 24 hours.

A good liquid medium (brain-heart infusion, tryptose broth) is tubed in 5 ml amounts, autoclaved, and stored in the cold. For each test, the tubes are numbered as indicated in Table V. Penicillin is diluted in phosphate buffer before the test to concentration of 5 units per ml (Solution I) and the required amounts are added to the tubes with a 1 ml pipette.

From the culture to be tested, well-grown but not older than 48 hours, 2 successive dilutions (1:100 and 1:10,000) are prepared in 2 tubes of the same medium used for the test (0.05 ml into 5 ml). 0.1 ml of the original culture, or of one of the dilutions, is then introduced into each tube as indicated in the table. For well-grown cultures, the inocula from the undiluted culture and from the two dilutions correspond respectively to 5×10^7 , 5×10^5 , and 5×10^3 organisms. The tubes are incubated and the results read

TABLE V
Test for Penicillin Resistance

			a <i>Staphylococcus aureus</i> NRRL Inoculum						b <i>Staphylococcus aureus</i> 26 Inoculum					
Units/ml	Penicillin Units/tube	Amts of Solution I ml/tube	0.1 ml of culture			0.1 ml of dilution 1:100			0.1 ml of dilution 1:10,000			0.1 ml of culture		
			Tube No			Tube No			Tube No			Tube No		
0.00	0.00	0	1	+		2	+		3	+		1	+	
0.05	0.25	0.05*	4	+		5	—		6	—		4	+	
0.20	1.00	0.20	7	—		8	—		9	—		7	+	
0.50	2.50	0.50	10	—		11	—		12	—		10	+	

* = 1 drop from a 1 ml pipette

TABLE VI
Test for Penicillin Resistance

		Penicillin			Inoculum			
		units/ml	0.1 ml of culture		0.1 ml of dilution 1:100		0.1 ml of dilution 1:10,000	
			Tube No		Tube No		Tube No	
a	<i>Staphylococcus albus</i> Shreffler	0.00	1	+	2	+	3	+
		0.05	4	+	5	+	6	+
		0.20	7	+	8	+	9	+
		0.50	10	+	11	+	12	+
		2.00	13	+	14	±	15	±
		5.00	16	+	17	—	18	—
b	<i>Staphylococcus albus</i> Cleaves	0.00	1	+	2	+	3	+
		0.05	4	+	5	+	6	±
		0.20	7	+	8	—	9	—
		0.50	10	+	11	—	12	—
		2.00	13	+	14	—	15	—
		5.00	16	+	17	—	18	—

after 24 hours a further reading after 48 hours may sometimes show some more positive results

Table V, a, shows the results for a typical sensitive strain, Table V, b, for a strain of medium resistance, Table VI for two penicillinase-producing strains. For resistant strains we generally repeat the test using amounts of penicillin ten times larger, as shown in Table VI. The amounts of resistant and sensitive cells in each strain are immediately evident by an inspection of the tables.

Discussion The results described in this paper indicate a definite influence of the size of the inoculum in penicillin sensitivity tests for staphylococcus. The importance of inoculum-size effects for penicillin activity has generally been belittled in the literature on the basis of some early observations,

mainly concerned with gross differences in sensitivity.⁷

The inoculum-size effects depends, first, on the presence in sensitive strains of a few mutant individuals of higher resistance, second, on the occurrence of strains producing penicillinase but consisting of cells individually sensitive.

The origin of the penicillinase-producing strains still awaits clarification. Since strains producing large amounts of penicillinase may consist of cells individually sensitive to penicillin, it seems unlikely that penicillinase production be secondarily acquired by resistant strains when grown in presence of penicillin.

⁷ Abraham, E. P., Chain, E., Fletcher, C. M., Gindler, A. D., Hestler, N. G., Jennings, M. A., and Florey, H. W., *Lancet*, 1941, **241**, 177.

The current tests for penicillin sensitivity⁸ do not take into account the inoculum size effects. The use of paper strip tests on heavily inoculated plates is likely to be misleading in cases of relatively sensitive strains producing penicillinase.

A simple test for penicillin resistance is proposed, which indicates the sensitivity of a staphylococcus culture, the presence and proportion of resistant individuals in it, and the degree of their resistance. The test also distinguishes resistant strains from relatively sensitive strains that produce penicillinase. This test may appear to be too delicate because of the fine differences in penicillin sensitivity which it reveals. It must be kept in mind, however, that concentrations of the order of 0.5 units per ml are almost the highest that can be obtained in the blood of patients. A reduction in blood level from 0.5 to 0.2 units per ml in the course of treatment may give a chance for growth to resistant bacterial cells present in a relatively sensitive strain. Moreover, the stepwise increase in bacterial resistance should lead us

⁸ Rummelkamp, C. H., and Maxon, T. *Proc. Soc. Exp. Biol. and Med.*, 1942, **51**, 386; Spink, W. W., and Ferris, V., *Science*, 1945, **102**, 221.

to consider any strain that is not very sensitive as a potential source of highly resistant variants. The therapeutic use of insufficient concentrations of penicillin may allow these variants to grow and spread, thus nullifying the usefulness of penicillin as a therapeutic agent.

Infections caused by penicillinase-producing strains consisting of individually sensitive cells may be susceptible to penicillin therapy if the number of bacteria present is small enough so that little penicillinase production occurs.

The use of similar tests for bacteria other than staphylococci should await more evidence regarding penicillin-fastness in those organisms.

Summary The results of penicillin sensitivity tests for staphylococci are influenced by the size of the inoculum on two accounts: (a) the presence in sensitive strains of a minority of resistant individuals originating by mutation, and (b) the occurrence of penicillinase-producing strains whose cells are individually sensitive to penicillin. A simple laboratory test is proposed for the detection and quantitative measurement of penicillin resistance in staphylococcus.

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Effect of Anesthetics and Convulsants on Brain Acetylcholine Content

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Whether acetylcholine plays an important role in central nervous system synaptic transmission has long been a matter of interest. It is known that central neural tissue contains the ester, can respond to its administration and can synthesize it. Though such facts, taken together with information on peripheral conduction, may suggest a transmitter role for acetylcholine in the central nervous system they do not prove it. Therefore, additional evidence has been sought, and one approach has been to attempt a correlation of acetylcholine content with activity.

Results have been varied. Thus, stimulation of afferent nerves has been found to increase production or liberation of acetylcholine by brain,^{1,2} but negative results have also been reported.^{3,4} Direct faradization

¹ Cortell, R., Feldman, J., and Gellhorn, E., *Am. J. Physiol.*, 1941, **132**, 588.

² Dikshat, B. B., *J. Physiol.*, 1934, **80**, 409.

³ Adams, H. M., McKim, R. A., Obrador, S., and Wilson, W. C., *J. Physiol.*, 1938, **93**, 45P.

⁴ Feldberg, W., and Schriever, H., *J. Physiol.*, 1936, **86**, 277.

of the isolated spinal cord of the rabbit⁵ and toad,⁶ and injection of KCl into the perfused cat brain⁷ have been shown to liberate the ester. It has been reported that prolonged strychnine convulsions elevate brain acetylcholine in the frog,^{1,8} but that neither strychnine, metrazol nor insulin convulsions raise it in the rabbit.⁹ In the rat, on the other hand, there are conflicting reports on the effect of hypoglycaemic convulsions on brain acetylcholine content.^{1,10} The effect of light on retinal acetylcholine in the frog has also been studied, and again divergent results have been obtained.^{11,12,13}

In a further attempt to correlate brain acetylcholine with activity we have measured, under constant conditions, the effect of two anesthetics (diffuse diminution of activity) and two convulsants (diffuse increase of activity) on brain acetylcholine content in the rat and frog.

Method Whole rat brain, including medulla, was placed in a weighed homogenizer tube (Potter-Elvehjem) containing 2 cc of ice cold, eserinized frog Ringer (NaCl 0.67 g, KCl 0.015 g, CaCl₂ 0.012 g, NaHCO₃ 0.015 g, eserine salicylate 10 mg, and water to 100 cc). The tube was reweighed and homogenization carried out in the original 2 cc from the fourth through the sixth minute after decapitation. After adding eserinized Ringer up to a total volume of 9 cc per gram of tissue, the whole was mixed for one min-

ute. The brei was then divided into two parts. An aliquot of one was assayed for free acetylcholine after centrifugation from the ninth to the twelfth minute at 3000 r.p.m. The remaining brei, acidified to about pH 4 with 0.5 N HCl, was placed in a boiling water bath for 2 minutes. When cool it was neutralized to phenol red, centrifuged, and assayed for total acetylcholine. Frog brain was similarly treated in a smaller homogenizer and made up to a final dilution of 10 cc per gram of tissue. It was assayed without centrifugation for total acetylcholine only.

The assay object was the eserinized, frog, rectus abdominis muscle mounted in a 4 cc muscle chamber. Emphasis has been laid on timing, since we have found that large discrepancies can result from irregular timing.

As we were interested primarily in any measurable relationship between acetylcholine content and activity, animals given convulsants were decapitated as soon as convulsions began. Thus, artefacts due to the anoxia of tonic seizures were avoided.

Results The data are shown in Tables I and II. On the average, free acetylcholine in rat brain was 67 and 35% higher after chloroform and nembutal anesthesia respectively than in the unanesthetized animal. Total acetylcholine was 35 and 30% higher than without anesthesia. In the frog, total brain acetylcholine was 20% elevated after nembutal. Neither free nor total acetylcholine was elevated in rats or frogs showing convulsive activity after picrotoxin or strychnine.

Our failure to find any effect of strychnine on frog brain acetylcholine conflicts with earlier findings of others^{1,8} who have reported an increase. On the other hand, strychnine has been claimed both to have no effect on rabbit brain acetylcholine¹ and to lower it.¹⁴ These conflicting data on the effects of strychnine may in part be the result of different degrees and duration of anoxia developed.¹⁰

The only data we have seen on the effect of anesthetics were incidentally reported as

⁵ Minz, B, *C. R. Soc. Biol., Paris*, 1936, **122**, 1214.

⁶ Li, T. H., *Chin. J. Physiol.*, 1938, **13**, 173.

⁷ Chute, A. L., Feldberg, W., and Smyth, D. H., *Quart. J. Exp. Physiol.*, 1940, **30**, 65.

⁸ Loewi, O., *Naturwiss.*, 1937, **25**, 173.

⁹ Chang, H. C., Hsieh, W. M., Li, T. H., and Lim, R. K. S., *Chin. J. Physiol.*, 1938, **13**, 153; Chang, H. C., Chiu, K. F., Hsu, C. H., and Lim, R. K. S., *Ibid.*, 1937, **12**, 1; Chang, H. C., Lim, R. K. S., and Lu, Y. M., *Ibid.*, 1938, **13**, 33; Chang, H. C., Chiu, K. F., Hsu, C. H., and Lim, R. K. S., *Ibid.*, 1938, **13**, 13.

¹⁰ Welsh, J. H., *J. Neurophysiol.*, 1943, **6**, 329.

¹¹ Easton, D. M., *Proc. Soc. Exp. Biol. and Med.*, 1945, **59**, 31.

¹² Lange, V., *Z. f. Physiol. Chem.*, 1943, **279**, 73.

¹³ Therman, P. O., *Acta Soc. Sci. Fenn., Ser. B*, 1938, **2**, 5.

¹⁴ Fegler, J., Kovarzik, H., and Lelusz-Lelchowiec, Z., *Klin. Wochschr.*, 1938, **17**, 240, 667.

TABLE I
Effect of Anesthetics and Convulsants on Brain Acetylcholine Content *

Acetylcholine in whole brain brev, μg per gram									
Rat					Frog				
Normal†	Chloroform	Nembutal	Picrotoxin	Strychnine	Normal	Nembutal	Strychnine	total	
free	total	free	total	free	total	free	total	total	
0.7	3.3	1.4	4.4			4.4	5.7	4.8	
0.6	3.0	0.9	3.9			5.4	6.3	6.6	
0.6	3.5	1.2	4.5			4.5	5.3	4.9	
0.6	2.1	0.8	2.7	0.6	2.5	5.4	6.2	4.8	
0.6		0.9				5.0	5.8	4.6	
0.6		0.8							
0.7		0.9							
0.5		0.9							
0.5		0.9							
0.6	3.7		0.9	4.9					
0.7	3.7		0.9	5.2					
0.7	3.3		1.0	3.9					
1.0	2.9		1.3	3.6	0.9	2.2	1.0	2.5	
1.0	3.1		1.5	3.3	1.0	2.0	1.1	2.6	
1.0	2.4				1.0	2.2			
0.7	2.3				0.4	2.2			
0.6	2.4						0.5	2.4	
0.5	2.5						0.9	2.4	
0.6	2.8						0.5	2.6	
—	—						0.6	2.8	

* Data on experimental animals are tabulated opposite controls run simultaneously.

† Average normal values, rat free 0.7 (S.E. 0.04), total 2.9 (S.E. 0.14).

TABLE II
Effect of Anesthetics and Convulsants on Brain Acetylcholine Content
Average Data and Statistical Significance *

Drug		Animals		Avg acetylcholine content, γ/g			
				Free		Total	
				content	difference	content	difference
Chloroform	10-60 min anesthesia	normal rats		0.6 ± 0.02		2.9 ± 0.31	
Nembutal	30 mg/kg 10-60 min anesthesia	anesthetized rats		1.0 ± 0.06	0.4 ± 0.07	3.9 ± 0.41	1.0 ± 0.51
Picrotoxin	20 mg/kg	anesthetized rats		0.8 ± 0.08		3.3 ± 0.15	
		normal rats		1.1 ± 0.12	0.3 ± 0.15	4.2 ± 0.37	0.9 ± 0.40
Strychnine	3 mg/kg	normal rats		0.9 ± 0.09		2.6 ± 0.19	
		convulsant rats		0.8 ± 0.12	0.1 ± 0.07	2.3 ± 0.09	0.3 ± 0.21
Nembutal	30 mg/kg	normal rats		0.7 ± 0.11		2.7 ± 0.13	
		convulsant rats		0.8 ± 0.11	0.1 ± 0.11	2.6 ± 0.07	0.1 ± 0.15
Strychnine	3 mg/kg	normal frogs		—	—	4.9 ± 0.21	
		anesthetized frogs		—	—	5.9 ± 0.18	1.0 ± 0.28
		normal frogs		—	—	4.9 ± 0.21	
		convulsant frogs		—	—	5.1 ± 0.11	0.3 ± 0.27

* Statistical significance is indicated as $\pm \text{S.E.}$, not as $\pm \text{P.E.}$

part of a different type of investigation¹⁰ Three nembutalized rats yielded an average value of 0.35 or 0.43 (depending upon method) micrograms of acetylcholine per gram of brain, whereas the figure for 7 normals was 0.25 or 0.35 μg per gram. Here the direction and magnitude of difference agree with our findings.

In general, our values for free acetylcholine

in the rat brain are somewhat higher than those reported elsewhere¹⁰ Preliminary experiments lead us to believe that the difference is probably a result of more efficient extraction because of finer homogenization obtained with the Potter-Elvehjem homogenizer than with the sand-in-mortar type used by others.

It is not yet possible to define rigorously

the physiological meaning of these results. One might assume that chloroform and nembutal raised brain acetylcholine above a "normal" level, but it is equally likely that the values obtained during anesthesia more nearly approximate the "true" resting levels. Thus greater activity whether that of the usual waking state or that of the more active, convulsive state, is paralleled by a lower brain acetylcholine content than is rest. Whether this reflects an increased rate of acetylcholine breakdown during activity cannot be said. The change observed might have been much greater if brain regions rather than whole brain had been assayed. As the experiments were done, a large change in a small region may have been masked by the diluent effect of a large volume of tissue in which acetylcholine content did not change.

It will be recalled that chloroform liberates free acetylcholine from bound precursor *in vitro*¹⁵. For this reason we also used the quite different type of anesthetic, nembutal. In two experiments *in vitro*, nembutal did not liberate free from bound acetylcholine in homogenized rat brain. For this reason, and because bound acetylcholine was actually increased more than the free after chloroform or nembutal, our *in vivo* results probably do not reflect simple liberation of free ester from bound precursor. It is always possible

that acetylcholine may accumulate as a result of esterase inhibition. Certain depressants have been shown to have anticholinesterase activity,^{2,16,17} but this is apparently not the case with chloroform or single large doses of barbiturate.¹⁸

Conclusions 1 Both free and total acetylcholine content of the whole rat brain are higher after nembutal or chloroform anesthesia (diffuse diminution of activity) than in the unanesthetized animal. The free acetylcholine change is more convincing after chloroform, whereas the total acetylcholine change is more convincing after nembutal. The total acetylcholine content of the frog brain is similarly elevated after nembutal.

2 Neither free nor total acetylcholine content of rat brain differed significantly after the onset of strychnine or picrotoxin convulsions (diffuse increase of activity) from that found in quiet animals awake. Nor did strychnine alter total acetylcholine content of the frog brain.

3 With the methods used, normal whole rat brain has been found to contain about 0.7 μ g of free and 2.9 μ g of total acetylcholine per gram wet weight, frog brain about 4.9 μ g of total acetylcholine per gram.

¹⁵ Bernheim, F., and Bernheim, M., *J. Pharm. and Exp. Ther.*, 1936, **57**, 427.

¹⁷ Eadie, G. S., *J. Biol. Chem.*, 1941, **138**, 597.

¹⁸ Adair, J., and Rovenstone, E. A., *Anesthesia and Analgesia*, 1941, **20**, 109.

¹⁵ Mann, P. J. G., Tennenbaum, M., and Quastel, J. H., *Biochem. J.*, 1938, **32**, 243.

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Effect of Nutrition on Susceptibility of Mice to Pneumococcal Infection

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Several workers have studied the effects of various diets on the susceptibility of mice and rats to induced pneumococcal infections.^{1,2,3,4} For the most part, these studies have dealt with the effects of B vitamin de-

ficiencies. The observed differences between control and experimental animals have been small, and not altogether consistent from laboratory to laboratory.

It has been found in these laboratories

¹ Wooley, J. G., and Sebrell, W. H., *Pub. Health Rep.*, 1942, **57**, 149.

² Robinson, H. J., and Siegel, H., *J. Infect. Dis.*, 1944, **75**, 127.

³ West, H. D., Bent, M. J., Rivera, R. E., and Tisdale, R. E., *Arch. Biochem.*, 1944, **3**, 321.

⁴ Davis, H. G., and McClung, L. S., *Proc. Soc. Exp. Biol. and Med.*, 1945, **59**, 37.

TABLE I
Effect of Diet and Concentration of Inoculum on the Resistance of Mice to Pneumococcal Infection

Diet	Culture concn	No of mice	No surviving at day indicated					Avg survival days	% survival
			1	2	3	4	6		
A*	10 ⁻⁸	24	24	20	16	15	13	4.2	54
	10 ⁻⁷	10	10	3	3	2	1	2.0	10
	10 ⁻⁶	56	56	18	11	10	6	1.91	11
	10 ⁻⁵	10	10	1	0	—	—	1.1	0
B†	10 ⁻⁶	10	10	6	4	4	4	3.2	40
C‡	10 ⁻⁶	10	8	8	6	6	3	3.4	30
Basal	10 ⁻⁶	52	52	50	48	46	45	5.50	86
	10 ⁻⁵	9	9	9	8	7	7	5.2	78
	10 ⁻⁴	14	14	13	13	13	13	5.6	93
	10 ⁻³	9	8	7	5	5	5	3.8	56
	10 ⁻²	10	7	3	3	3	3	2.2	30

* Purina Fox Chow

† Rockland Mouse Diet

‡ Stock diet of Robinson and Siegel²

TABLE II

Duration of Dietary Regimen and Change in Susceptibility of Mice to Pneumococcal Infection

Days on basal diet*	No of mice	No surviving 6 days	Avg survival time, days
0	10	4	3.0
1	10	6	4.5
4	10	8	5.2
5-16	52	45	5.5

* Previous to injection. After injection all animals were maintained on the basal diet.

Dose = 10⁻⁶ ml culture per mouse

that a tremendous difference in susceptibility to pneumococcal infection exists between mice which have been fed a purified diet and mice maintained on a commercial small-animal diet.

Albino mice (18 to 24 g) were injected intraperitoneally with 0.5 ml of a dilution of a 17 hour culture representing 10 ml of a 10⁻⁶ concentration. The actual number of organisms injected varied from 235 to 700 as determined by plate counts. The pneumococcus, Type I, SV-1 strain, was grown on proteose-peptone-blood broth, and was passed through mice at weekly or bi-weekly intervals.

The basal diet consisted of casein 180, salts 20, cotton-seed oil 30, cod liver oil 20 and sucrose 750 g, supplemented by thiamin chloride hydrochloride 5, riboflavin 5, pyri-

doxine chloride 2, calcium pantothenate 20, nicotinamide 40, p-aminobenzoic acid 20, 2,3-dimethyl-1,4-naphthoquinone 0.2 and α -tocopherol 1 mg and choline chloride 1 g per kilo of diet respectively.

Mice not on experiment were maintained on commercial diet A (Table I). Experimental mice usually were fed the diet to be tested for 5 days before the injection of the pneumococci. Experimental animals were observed over a 6 day period, and the results were recorded in terms of average survival time (maximum 60).

Results Table I shows the effect of various dosages of pneumococcus culture on the production of infection in mice which had been maintained on different diets. It is seen that on diet A the mice are quite susceptible to infection, and that it is only when the culture is very dilute (10⁻⁸) with an average dose of 3.5 organisms, that any considerable proportion survives the injection. A control group maintained on this diet, was injected with the culture used for each experiment. The data of Table I (dose 10⁻⁶ ml) represent the summation of 7 experiments with groups of 5 to 10 mice which gave average survival times ranging from 1.4 to 3.0 days and from 0 to 20% survival at the termination of the experiment. On diet B the mice appear to be somewhat less sus-

TABLE III
Protocol of Exp 5, May 29, 1945

Diet	No of mice	No surviving at day indicated					Avg survival days
		1	2	3	4	6	
A*	10	10	1	1	1	0	1.4
Basal	10	10	10	8	8	7	5.0
Biotin, inositol, folic acid	10	10	10	9	9	9	5.6
10% brewer's yeast	10	10	9	9	8	5	4.6
5% 70 A I liver	10	10	9	7	7	6	4.5
Alcoholic extract of liver	10	10	10	9	9	9	5.6

* Purina Fox Chow

ceptible, the average survival time, 3.2 days, is greater than any found with the same dosage for a group of 10 mice on diet A. Mice on the stock diet of Robinson and Siegel² are less susceptible but significantly more susceptible than those on the purified diet.

Mice maintained on the basal diet are very resistant to pneumococcal infection. Thus it was only when the concentration of pneumococcus culture reached 10^{-2} , the equivalent of about one million lethal doses for mice on diet A, that any considerable proportion succumbed to the infection. A control group of 5 to 10 mice, maintained on basal diet, was injected with the culture used for each experiment. The data of Table I (dose 10^{-6} ml) represent the summation of 8 such experiments which gave average survival times ranging from 4.4 to 6.0 days and from 60 to 100% survival.

The protocol of a representative experiment is given on Table III. All animals were injected with 0.5 ml aliquots of the same culture dilution representing 10^{-6} ml of a 17 hour blood broth culture. Animals were caged in groups of 5, with 2 groups on each diet. Injections were completed within 45 minutes. However, to distribute evenly the effect of any possible change in the culture during this period, one group of five on each of the diets was inoculated before the second group on any diet was injected. The composition of the diets is given in Table IV.

The time of pretreatment on the basal diet to effect a change in susceptibility was determined in the experiments reported in Table II. It appears that if the mice are changed from diet A to the basal diet at the

time of injection some increased resistance to the infection may result, while even a single day on the basal diet definitely increases the resistance.

In Table III are shown the results of a number of experiments designed to test the effect of various dietary supplements to the basal diet. In every instance the average survival time of the group of mice fell within the range of survival times found for groups of 5 to 10 mice on the basal diet. In only one instance, that of the diet in which 5% of the 70% alcoholic insoluble fraction of liver was incorporated, was the average survival time low enough to indicate that further investigation would be desirable. It is apparent that the difference between the basal and the commercial diet cannot be attributed to any known vitamin or, in fact to any known dietary essential.

Discussion. A number of possible explanations exist for the difference in susceptibility to pneumococcal infection of mice on the two types of diet. These theories fall into two main groups. Either a protective factor in the purified diet or a factor in the commercial diet which increases the susceptibility might be postulated. The first appears unlikely. The latter explanation would be best fitted to explain the results to date. On this hypothesis some factor essential to the rapid multiplication of the pneumococcus, but not necessarily an absolute requirement for growth, would be absent from, or in low concentration in the synthetic diet. This hypothetical substance (or substances), would not be essential for the mouse. It would, in fact, be present in minimal concentration in the blood and body fluids of

TABLE IV

Effect of Various Supplements to the Purified Diet on Susceptibility of Mice to Pneumococcal Infection

Supplement to basal diet	No of mice	No surviving 6 days	Avg survival time, days	% survival
Biotin, inositol*	5	5	6.0	100
Biotin, inositol, folic acid†	20	18	5.7	90
Alcoholic extr of liver‡	10	9	5.6	90
75% corn starch	11	8	5.2	73
3% salts	6	6	6.0	100
12% casein	10	8	5.3	80
12% proteose peptone	10	9	5.7	90
2% 70 A I liver§	10	10	6.0	100
5% 70 A I liver	10	6	4.5	60
10% brewer's yeast	20	15	5.3	75
5% asparagine	10	10	6.0	100

Each supplement was incorporated into the basal diet by replacement of an equal weight of sucrose

* 40 mg inositol, 400 μ g biotin per kg diet

† 2 mg biotin, 80 mg inositol, and 15 g folic acid concentrate (equivalent to 6 mg of folic acid $\pi = 40,000$) per kg diet

‡ Fraction soluble in 70% alcohol equivalent to 0.8 g liver per g diet

§ Fraction insoluble in 70% alcohol

Dose = 10⁻⁶ ml culture per mouse

the mouse, except insofar as higher levels were maintained by a more or less continuous supply from exogenous sources. Such an hypothesis would explain the rapid change in susceptibility of the mouse which occurs when the dietary regimen is changed.

The conclusions to be drawn from the present experiments may or may not be applicable to other types and strains of pneumococci. However, Robinson and Siegel² found mice on all purified diets to be less susceptible to pneumococcal infection than those on their stock diet. The data reported here show similar comparative effects from their stock diet and the purified diets. It seems likely, therefore, that the same factors influencing

susceptibility may be operative in both laboratories.

Summary The resistance of mice to the intraperitoneal injection of Type I pneumococcus was found to be markedly dependent on the type of diet on which they were maintained. Those on a purified diet survived the equivalent of 100,000 lethal doses for those on a commercial laboratory diet. This difference could not be ascribed to any known dietary essential.

We are indebted to Armour Laboratories for generous supplies of alcoholic liver extract and folic acid concentrate, and to Dr Marion B. Sherwood for plate counts, dilutions, and the care of cultures.

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Further Purification of the Follicle-Stimulating Hormone and Its Effect in Normal and Hypophysectomized Rats *

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Follicle-stimulating hormone preparations have been obtained previously by the diges-

tion of an aqueous extract of sheep pituitary powder with trypsin.^{1,2} The extracts ob-

* Supported in part by a grant from the Wisconsin Alumni Research Foundation, and in part by grants from Eli Lilly and Company and The Abbott Laboratories.

¹ McShan, W. H., and Meyer, Roland K. *J. Biol. Chem.* 1938, **126**, 361.

² McShan, W. H., and Meyer, Roland K. *J. Biol. Chem.*, 1940, **135**, 473.

tained by this method were biologically tested mainly by use of normal immature female rats, although the effect of a few of these preparations in hypophysectomized rats was reported¹ The preparations obtained by the method reported in 1940² when used in the human being caused local reactions at the site of injection, which made it necessary to do further work in order to remove this local reacting factor This report is concerned with the method of removal of this factor, and with the assay of several preparations in normal and hypophysectomized rats

Experimental Removal of local reacting factor The procedure followed is the same as that reported previously² until the fraction of the digest which is soluble at pH 4 is obtained Instead of precipitating the active material at this stage with ethyl alcohol, it was dialyzed in the cold against distilled water which was changed 5 times This caused the formation of a light inactive precipitate which was separated by centrifuging The hormone was precipitated from the solution by addition of 4 volumes of 95% alcohol Precipitation was aided by addition of 4 drops of 2N HCl while the mixture was being stirred

The precipitate was recovered by centrifuging and the active material was recovered by extracting the precipitate with cold distilled water It was necessary to make at least 5 extractions using 75 ml of water each time for each 200 g of pituitary powder Thorough extraction was accomplished by shaking the mixture with glass beads During the third extraction the insoluble residue became gummy so that it was necessary to break the insoluble particles with a glass rod Each extraction was continued for 2 to 4 hours followed by centrifuging and decantation of the extract into 1.5 liters of cold acetone After each extraction the extract was added to the acetone to which the others had been mixed A few drops of 2N HCl were added followed by stirring to cause precipitation after which the mixture was allowed to stand overnight in the cold

The precipitate was collected by centrifuging It was dried either by washing

with acetone or by lyophilizing after solution in 0.02 M phosphate buffer of pH 7.4 The product obtained by either of these methods of drying is soluble in water, or in 0.9% saline and can be sterilized by Seitz filtration

Assay—Normal rats The total amount of the hormone preparation to be given to a single rat was dissolved in 4.5 ml of 0.9% sodium chloride Solutions of this kind were injected subcutaneously into 21 day old female rats in nine injections of 0.5 ml each beginning on the afternoon of the first day and followed by morning and afternoon injections for the next 4 days Autopsy was done during the morning of the sixth day The ovaries were removed, examined grossly with the aid of transmitted light for the presence of follicles and corpora lutea and weighed These ovaries were prepared for microscopic study and later they were examined for the presence of lutein tissue

Hypophysectomized rats Immature female rats 28 days old were obtained from Sprague-Dawley, Inc These animals were hypophysectomized on the 29th day, and the injections were started the following day when they were 30 days old The total amount of hormone to be given each rat, which was usually 2.5 g,[†] was dissolved in 10 ml of 0.9% sodium chloride They were injected subcutaneously with 0.5 ml on the morning and afternoon of each day for 10 days, and were killed on the morning of the eleventh day at which time the ovaries were removed, examined, weighed and prepared for histological study Completeness of hypophysectomy was determined at the time of autopsy by use of a binocular microscope

Results and Discussion Assay of preparations that contained the local reacting factor in normal and hypophysectomized rats The 10 preparations listed in Table I were prepared according to the tryptic digestion method reported in 1940,² and thus contained the local reacting factor Five hun-

[†] Indicates in all cases grams equivalent of original pituitary powder used in making the preparation

TABLE I
Assay of FSH Preparations Which Caused Local Reactions in the Human Female*

Prep No	Mg per g eq	Total† dose, g	No of rats	Wt of ovaries, mg	Qualitative response	
					Ovaries	Vagina
Normal rats injected 45 d vs						
5000†	11 57	0 5	6	85	follicles only	O‡
508	15 10	0 5	6	56	" "	No
514	—	0 5	3	27	" "	No
		1 0	3	99	" "	O
515	13 50	0 5	3	32	" "	No
516	12 40	0 5	6	50	" "	O
517	8 33	0 5	6	62	" "	O
519	—	0 5	6	105	" "	O
520	—	0 5	3	38	" "	No
		1 0	3	100	" "	O
522	11 80	0 5	7	41	" "	No
523	13 24	0 5	3	110	corpora	O
Avg	12 13					
Hypophysectomized rats injected 10 d vs						
5000†	11 57	2 5	4	183	follicles only	O
508	15 10	2 5	5	232	" and few bl pts	O
514	—	2 5	5	65	" only	O
516	12 40	2 5	5	263	" "	O

* Made by method reported in 1940 2

† FSH5000 was made by combining parts of preparations 508, 510, 513, 515, 516, and 517

‡ Indicates g equivalent of pituitary powder

§ O indicates that vagina was open, No that vagina was not open

dred grams of pituitary powder was used in making each of these preparations. The total dose used for assay in normal rats in most cases was 0.5 g. The ovarian weights obtained by injecting this amount of hormone weighed from 27 to 110 mg and the average was 61 mg. These ovaries, with the exception of those produced by preparation 523, contained follicles only as indicated by microscopic examination at the time of autopsy. The uteri were highly distended in all cases, and certain of the preparations caused the vaginas to open. The fact that preparation 523 produced ovaries that contained corpora lutea suggests that the extract was not properly digested. The failure of the extract to clear during digestion as it usually does, further supports this idea, and emphasizes the need for making a systematic study to determine the optimum conditions for digestion.

Preparations 5000, 508, 514 and 516 were tested in hypophysectomized rats by administering a total dose of 2.5 g over a period of 10 days. The ovaries obtained ranged in weight from 65 to 263 mg, the average weight was 186 mg. The uteri were distended with fluid and the vaginas were open in all

cases. Macroscopic examination of these ovaries showed that they contained only follicles with the exception of those from one rat injected with FSH508 which contained a few hemorrhagic follicles.

It was found on microscopic examination that certain of these ovaries contained several patches of hypertrophied theca cells. One ovary contained some lutein cells, and there were a total of 8 small or partly formed corpora in four ovaries. Thus these preparations as indicated by assays in hypophysectomized rats contained very little luteinizing hormone but evidently were not completely devoid of luteinizing activity.

Assay of preparations that were free of the local reacting factor in normal and hypophysectomized rats. The results of the assays in normal rats of 15 different follicle stimulating preparations that were essentially free of the local reacting factor, are given in Table II. These preparations were tested by administration of 0.5 g and 1 g total doses. The ovaries as indicated by macroscopic examination at the time of autopsy contained only follicles with the exception of 3 pairs produced by 3 different preparations each given in a total dose of one gram. The

tained by this method were biologically tested mainly by use of normal immature female rats, although the effect of a few of these preparations in hypophysectomized rats was reported¹. The preparations obtained by the method reported in 1940² when used in the human being caused local reactions at the site of injection, which made it necessary to do further work in order to remove this local reacting factor. This report is concerned with the method of removal of this factor, and with the assay of several preparations in normal and hypophysectomized rats.

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clear that this preparation contained very little luteinizing activity as indicated by the administration of a total dose of 2.5 g over a period of 10 days. This demonstrates that follicle-stimulating preparations of a high degree of physiological purity can be obtained by the tryptic digestion method as described in this report.

The criteria used in assaying follicle-stimulating preparations obtained by the tryptic digestion method are relatively large increases in the weights of the ovaries of normal and hypophysectomized rats without the production of any lutein tissue or discrete corpora lutea. If these rigid specifications are to be the basis for determining the absolute purity of follicle-stimulating preparations then not all preparations obtained by tryptic digestion are physiologically pure. These preparations are suitable for many practical purposes as demonstrated by their use in human beings³ and in laboratory and domestic animals.^{4,5,6} The large doses and long injection periods used in testing the preparations are believed to be suitable for the maximum expression of any small amount of luteinizer that might be present in the preparations. When intact immature rats were used for assay most of our preparations have produced ovaries that contained only follicles as indicated by macroscopic examination. We believe that results of this kind when obtained consistently, indicate that this method in which intact rats are used is as sensitive for detecting small amounts of luteinizer in the presence of follicle-stimulating hormone as are hypophysectomized rats. This is because the intact rat has a normal endocrine balance and the secretion of luteinizer by the animal's own pituitary gland would be added to any traces present

in the follicle-stimulating preparation injected. If under these conditions a preparation produces ovaries that contain only follicles it is believed the preparation has a high degree of physiological purity.

The product obtained by the tryptic digestion process is undoubtedly different chemically from preparations prepared by other methods such as salt fractionation reported by Greep *et al.*^{7,8} It is also probable that the product obtained by the method developed by these workers is different in its physiological action from that obtained by tryptic digestion as little increase in ovarian weight resulted and no secretion of estrogen was obtained even when large doses were given to hypophysectomized rats.⁹ Another difference is that these workers used fresh hog pituitary glands in their work on chemical fractionation while whole sheep pituitary powder was used in the tryptic digestion method. Further study is needed to determine whether these variables are responsible for the different physiological effects produced by the two kinds of preparations.

Summary. A method is described for the preparation of follicle-stimulating preparations by tryptic digestion and the removal of a fraction which caused reactions in the human being at the site of injection.

Preparations made by the tryptic digestion method before and after it was changed to remove the local reacting factor were assayed both in normal and hypophysectomized rats. By macroscopic observation the ovaries from both kinds of rats appeared to contain only follicles but microscopic examination showed that a few contained lutein tissue indicating that not all the preparations were entirely devoid of luteinizing activity.

⁴ Casida, L. E., Meyer, R. K., McShain, W. H., and Wisniewski, W. *Am. J. Vet. Res.*, 1943, 4, 76.

⁵ Warwick, E. J., Murphree, R. L., Casida, L. E., and Meyer, R. K. *Anat. Rec.*, 1943, 87, 279.

⁶ Casida, L. E., Warwick, E. J., and Meyer, R. K. *J. Animal Science*, 1944, 3, 22.

⁷ Greep, R. O., Van Dyke, H. B., and Chow, B. F. *Endocrinology*, 1942, 30, 635.

⁸ Chow, B. F., Van Dyke, H. B., Greep, R. O., Rothen, A., and Sheddorsky, T. *Endocrinology*, 1942, 30, 650.

⁹ Greep, R. O., Van Dyke, H. B., and Chow, B. F., *J. Biol. Chem.* 1940, 133, 289.

TABLE II
Results of the Assay in Normal and Hypophysectomized Rats of Follicle stimulating Preparations *

Prep No	Total dose, g eq	No of rats	Wt of ovaries	Qualitative response	
				Ovaries	Vagina
230	1.0	3	87	follicles only	O†
234	0.5	6	38	" "	No
234	1.0	6	90	" "	O
235	0.5	6	50	" "	O
235	1.0	3	90	" "	O
236	0.5	3	68	" "	O
236	1.0	3	90	" "	O
				(few CL in 1 pair ov)	
237	0.5	6	35	follicles only	No
320	0.5	3	42	" "	No
321	0.5	3	35	" "	No
321	1.0	3	104	" "	No
322	0.5	6	46	" "	No
322	1.0	3	55	" "	No
323	0.5	3	34	" "	No
323	1.0	9	60	" "	No
				(1 pair ov weighed 114 mg, contained few CL)	
327	0.5	6	61	follicles only	No
327	1.0	3	102	" "	O
				(few CL in 1 pair ov)	
328	0.5	9	32	follicles only	O
328	1.0	15	75	" "	O
330A	0.5	3	41	" "	
330B	0.25	6	34	" "	No
330B	0.5	9	68	" "	O
331	0.5	3	36	" "	No
331	1.0	3	69	" "	No
333	0.5	6	25	" "	No
333	1.0	7	57	" "	No
				Hypophysectomized Rats	
			(2.5 g given over 10 days)		
333	2.5	4	75	follicles only	O

* These preparations were made after the method was changed to remove the local reacting factor and several of them were administered to human clinical cases.³

† O indicates that vagina was open, No that vagina was not open.

uteri were usually distended with fluid and in many cases the vaginas were not open. It is of interest to note that 0.25 g of FSH330B produced ovaries that had an average weight of 34 mg which was almost as great as that obtained with 0.5 g of the other preparations. This difference is believed to be due to the fact that the aqueous extract used in making FSH330B was not supercentrifuged before it was digested with trypsin. This suggests that more active preparations may be obtained without supercentrifuging but further work is necessary to test this possibility. Several of these preparations were effective in stimulating the ovaries of the human female, and in addition they caused little trouble from the

standpoint of producing local reactions at the injection sites.³

FSH333 was assayed by use of hypophysectomized rats. The average weight of the ovaries was 75 mg and they consisted of follicles only as indicated by observation at the time of autopsy (Table II). These ovaries were sectioned and examined microscopically. One pair of ovaries contained no lutein cells, another pair showed one small group of hypertrophied theca interna or granulosa cells, the third pair contained 3 small groups of lutein cells, and the fourth pair had 5 very small corpora. Thus it is

³ Davis, M. Edward, and Hellbaum, Arthur A., *J. Clin. Endocrinology*, 1944, 4, 400.

clear that this preparation contained very little luteinizing activity as indicated by the administration of a total dose of 2.5 g over a period of 10 days. This demonstrates that follicle-stimulating preparations of a high degree of physiological purity can be obtained by the tryptic digestion method as described in this report.

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Urinary Excretion of Estrogens and 17-Ketosteroids in Young, Adult Males With Infectious Hepatitis

HELENA GILDER AND CHARLES L. HOAGLAND *

From the Hospital of The Rockefeller Institute for Medical Research, New York

Male subjects with chronic diseases of the liver frequently display symptoms which are best explained by assuming an increased concentration of circulating, biologically active estrogenic compounds. Testicular atrophy,¹ gynecomastia, decrease in total body hair, development of spider telangectasia, feminine distribution of pubic hair, and loss of libido,^{2,3,4} include some of the phenomena associated with cirrhosis of the liver in the male. In some of these cases a high output of urinary estrogen has been recorded, along with a fall in the total excretion of urinary androgens.

Numerous experiments with animals have shown that the liver inactivates, or otherwise disposes of large quantities of exogenous and endogenous estrogens.⁵⁻¹¹ If estrogenic compounds are injected into the portal circulation, their systemic effects are negligible compared

to the effect of a similar quantity of hormone injected into extra-portal areas.^{12,13,14} At least 2 major roles have been assigned to the liver in the metabolism of estrone, one, that it is rapidly effective in converting estrone to estradiol, and secondly, that it acts to convert all estrogenic materials to compounds which are no longer phenolic in character.⁹ These data provide a theoretical basis for explanation of the marked degree of feminization occasionally seen in males with advanced cirrhosis of the liver^{15,16} and suggest that estrogen metabolism may be significantly impaired in chronic hepatic insufficiency.

In the present study an attempt has been made to learn whether defective estrogen metabolism likewise occurs in acute infectious hepatitis. At the same time studies on the urinary excretion of the total 17-ketosteroids have been carried out in order to learn if the metabolism of androgens may also be affected in acute liver insufficiency. The results of serial studies of the urinary excretion of estrogens and 17-ketosteroids in 11 males with infectious hepatitis are recorded in the accompanying graph (Fig 1). All patients selected for this study were young adult males, previously in good health, and with no demonstrable diseases other than hepatitis. The number of days following the appearance of the first symptoms are recorded on the abscissa. The concentration of free urinary estrogen and total 17-ketosteroids, in micrograms and milligrams per 24 hours, respec-

* The authors wish to acknowledge the valuable technical assistance of Miss Elizabeth Van Pelt.

1 Morrison, T. G., *Arch. Path.*, 1944, **37**, 39.

2 Edmondson, H. A., Glass, S. J., and Soll, S. N., *Proc. Soc. Exp. Biol. and Med.*, 1939, **42**, 97; Glass, S. J., Edmondson, H. A., and Soll, S. N., *Endocrinology*, 1940, **27**, 749.

3 Glass, S. J., Edmondson, H. A., and Soll, S. N., *J. Clin. Endocrinology*, 1944, **4**, 54.

4 Sheldon, J. H., *Lancet*, 1934, **2**, 1031.

5 Zondek, B., *Skandinav. Arch. f. physiol. Chem.*, 1934, **70**, 133.

6 Heller, C. G., and Heller, E. J., *Endocrinology*, 1943, **32**, 64.

7 Talbot, N. B., *Endocrinology*, 1939, **25**, 601.

8 Biskind, M. S., and Biskind, G. R., *Endocrinology*, 1942, **31**, 109.

9 Schiller, J., and Pincus, G., *Endocrinology*, 1944, **34**, 203.

10 Longwell, B. B., and McKee, F. S., *J. Biol. Chem.*, 1942, **142**, 757.

11 Israel, S. L., Meranze, D. R., and Johnston, C. G., *Am. J. Med. Sci.*, 1937, **194**, 835.

12 Golden, J. B., and Sevinghaus, E. L., *Proc. Soc. Exp. Biol. and Med.*, 1938, **39**, 361.

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14 Segaloff, A., *Endocrinology*, 1943, **33**, 209.

15 Kyrle, J., *Verhandl. d. deutsch. path. Gesellsch.*, 1909, **13**, 391.

16 Weichselbaum, A., *Verhandl. d. deutsch. path. Gesellsch.*, 1910, **14**, 234.

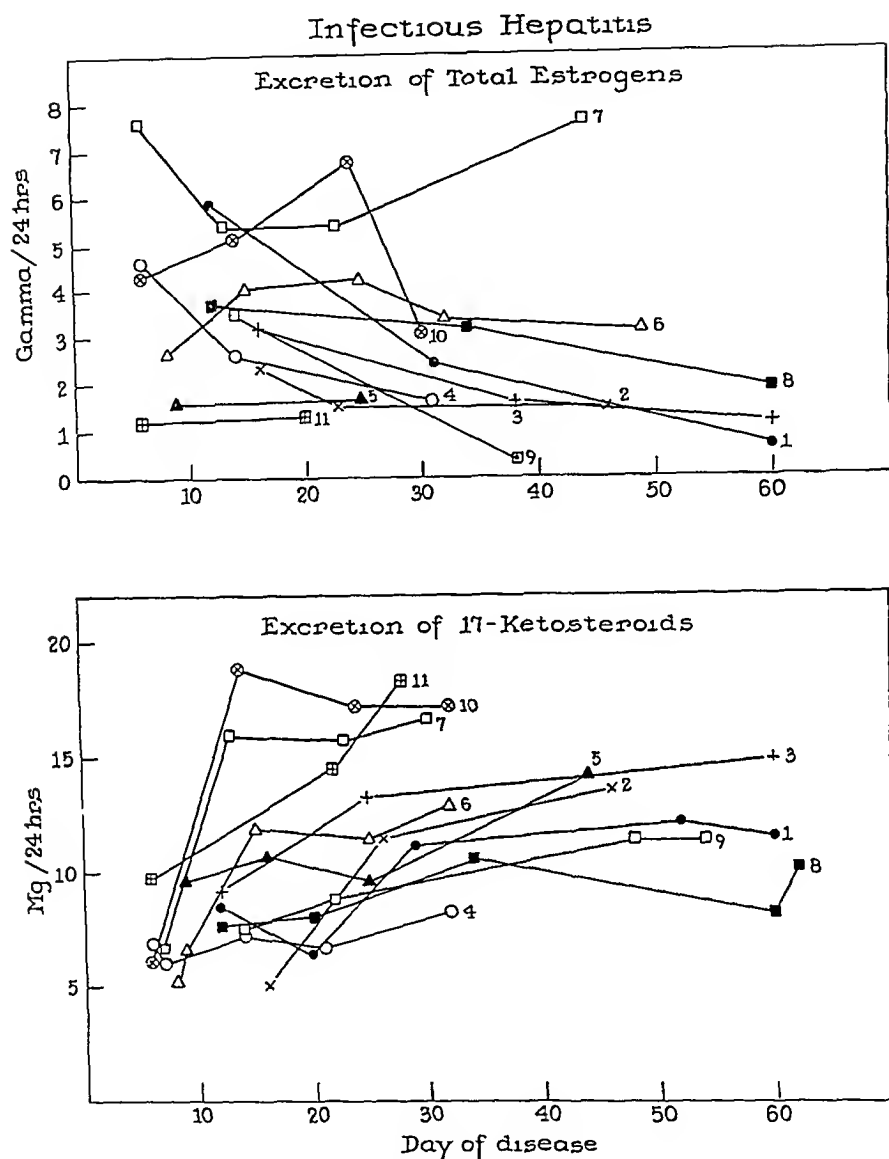


FIG 1

Graph showing values for the urinary excretion of estrogens and 17 ketosteroids in young, adult males with infectious hepatitis

tively, has been plotted on the ordinate. The estrogen assays were carried out by a modification of the Allen-Doisy technic,^{17,18} in which

¹⁷ Thayer, S. A., Doisy, E. A., Jr., and Doisy, E. A., *Yale J. Biol. and Med.*, 1944, **17**, 19

¹⁸ Allen, E., and Doisy, E. A., *J. Am. Med. Assn.*, 1923, **81**, 819

0.1 cc of a urine extract prepared by hydrolysis, extraction with carbon tetrachloride, and concentration of the phenolic compounds in the standard manner, was injected 4 times in 48 hours into spayed mice, and vaginal smears studied on the third day. An average of 15 animals were used for each urine extract. The method for the 17-ketosteroids was essentially

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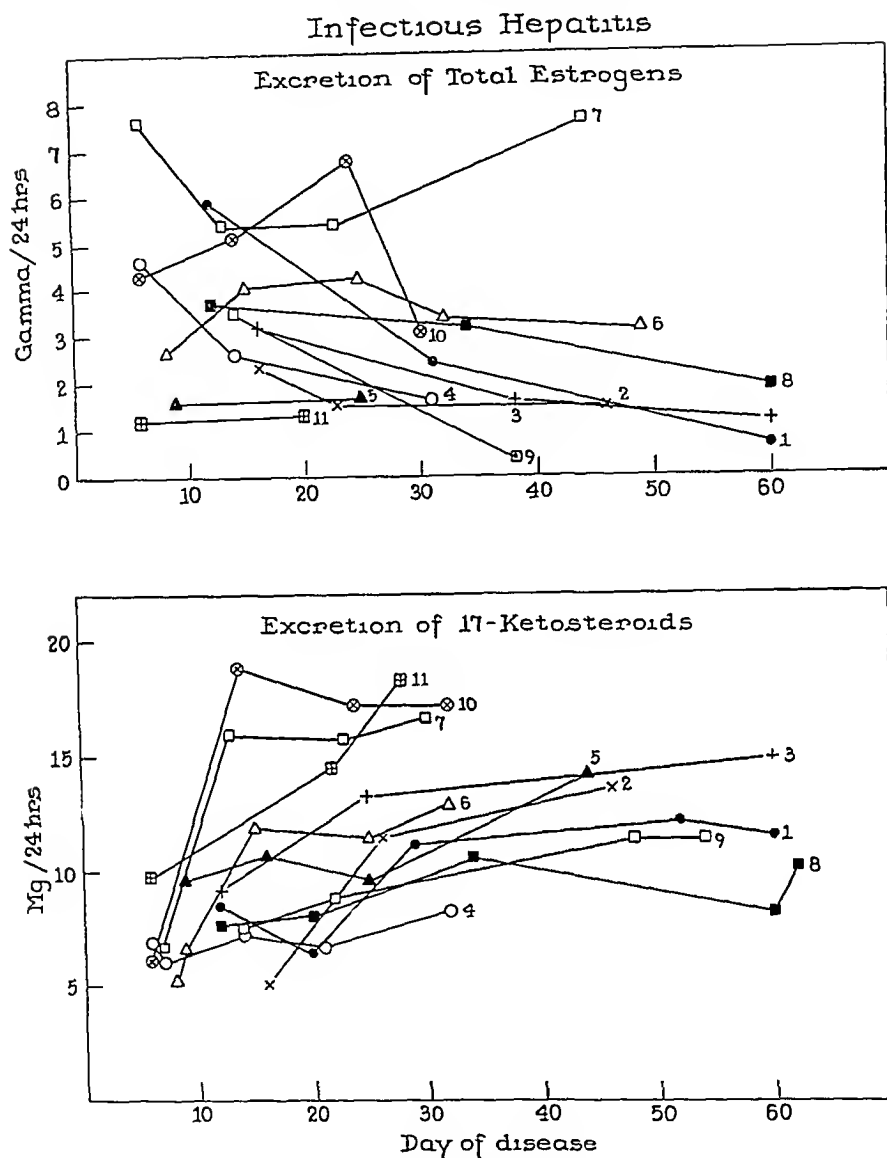


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tively, has been plotted on the ordinate. The estrogen assays were carried out by a modification of the Allen-Doisy technic,^{17, 18} in which

¹⁷ Thayer, S. A., Doisy, E. A., Jr., and Doisy, E. A., *Yale J. Biol. and Med.*, 1944, **17**, 19

¹⁸ Allen, E., and Doisy, E. A., *J. Am. Med. Assn.*, 1923, **81**, 819

0.1 cc of a urine extract prepared by hydrolysis, extraction with carbon tetrachloride, and concentration of the phenolic compounds in the standard manner, was injected 4 times in 48 hours into spayed mice, and vaginal smears studied on the third day. An average of 15 animals were used for each urine extract. The method for the 17-ketosteroids was essentially

that of Talbot,¹⁹ with modifications suggested by Dr Ephraim Shorr.²⁰ In general the methods proved to be reliable, and the results reproducible.

The range of excretion of estrogens in normal males is low, and quite varied. Moreover, the values depend to a great extent upon the technic employed for the preparation of the urinary extract and upon the method used for assay. It was decided, therefore, to follow the estrogen excretion of each patient at frequent intervals throughout the course of the disease, and to use as a control base line for each case the quantity of estrogenic substances excreted following recovery from hepatitis.

With the convalescent level as a control, it may be seen that in nearly every case there was a significant rise in estrogen excretion during the acute phase of hepatitis, and that a diminishing elevation in estrogen excretion was maintained until relatively late in convalescence. In Subject 7, values for the urinary excretion of estrogens were high within the first 5 days following the onset of the disease process. By the 12th day an abrupt fall had occurred, associated with marked clinical improvement. No further drop in estrogen excretion was observed. However, on the 45th day after onset of the disease, a significant rise in the excretion of urinary estrogens had again occurred, coincident with a return of clinical signs of hepatitis. Following clinical recovery of the patient, normal values were recorded for the urinary excretion of estrogens of 2.9 μg per 24 hours. Delayed clinical recovery in another patient with hepatitis, Case No. 6, was likewise reflected in a persistent moderate elevation of urinary estrogens. Further studies, attempting to relate persistent elevation in urinary estrogen excretion to delay in convalescence and chronicity in infectious hepatitis are under way, and the results will be published subsequently.

In most cases there appeared to be a significant correlation between the severity of the

attack of acute hepatic insufficiency, as judged from the results of clinical and laboratory studies, and the estrogenic activity of the urine. For example, Case No. 5 and No. 11, in whom normal values for the urinary excretion of estrogens were recorded during the course of the disease, presented exceptionally mild symptoms of infectious hepatitis requiring only 20 and 28 days, respectively for complete clinical recovery.

The normal range of the urinary level of 17-ketosteroids in males, like that for the estrogens, is variable, with most reports placing values at 8-28 mg per 24-hour period of urinary excretion. Again because of the wide variation in the normal level of androgen excretion, it was considered that the convalescent level of 17-ketosteroids provided the best possible normal base line for each patient, and the most effective standard for a comparison of the degree of aberration in 17-ketosteroid excretion occurring in each case during the acute and convalescent states of infectious hepatitis.

The urinary excretion of 17-ketosteroids in the majority of cases was low in the early stages of hepatitis, and rose to a normal level during the period from the 20th to the 50th day of the disease, where it remained approximately constant. In some cases the level of excretion was more than twice the value obtained during the acute stages of the disease. In 2 cases in whom there occurred recrudescence of hepatitis with a return of clinical symptoms, the 17-ketosteroid levels of the urine fell again to values comparable to those observed during the first acute attack of the disease. In both cases clinical recovery was followed by an elevation of the urinary 17-ketosteroids to normal values.

At first glance there appears to have been a reciprocal relationship between the levels of excretion of estrogens and 17-ketosteroids in the urine of patients with acute hepatic insufficiency. Closer analysis, however, reveals that in all cases, a rise in 17-ketosteroids, often to a normal level, occurred considerably in advance of a significant decrease in the level of urinary estrogens. The excretion of 17-ketosteroids is temporarily depressed in a variety of conditions, including fasting protein

¹⁹ Talbot, N. B., Berman, R. A., MacLachlan, E. A., and Wolfe, J. K., *J. Clin. Endocrinology*, 1941, **1**, 668.

²⁰ Shorr, E. P., personal communication.

depletion, and general debility. It is quite possible, therefore, that the depression of the excretion of 17-ketosteroids observed in acute hepatic insufficiency may have resulted from the secondary effects of the disease, rather than to a rise in the level of active circulating estrogens. That reciprocal depression of androgen formation may occur eventually, as a result of the failure of the liver to accomplish the inactivation of estrogenic compounds, is a definite possibility, and may conceivably account for the persistent diminution in the excretion of 17-ketosteroids which has been observed in chronic hepatic insufficiency.

Summary The levels of excretion of estrogens and 17-ketosteroids in the urine have been determined in 11 young adult males with

acute infectious hepatitis, at frequent intervals, from onset of the disease to convalescence. In 9 moderately severe cases there was a significant elevation in the level of excretion of biologically active estrogens in the urine which persisted at a slowly diminishing elevation until convalescence was considerably advanced. There was no significant increase in the concentration of the urinary estrogens in 2 cases of hepatitis which presented only mild clinical symptoms.

Marked to moderate depression occurred in the level of excretion of urinary 17-ketosteroids early in the course of acute hepatitis, with a return to normal values considerably in advance of the period of diminished estrogen excretion and of clinical recovery.

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Adequacy of the Known Synthetic Vitamins for Normal Feathering and Pigmentation in Chicks

DOUGLAS V. FROST, F. PEIRCE DANN, AND FLOYD C. MCINTIRE
(Introduced by Carl Nielsen)

From the Abbott Laboratories, North Chicago, Ill.

The existence of a factor, or factors, in liver or yeast other than pantothenic acid, *p*-aminobenzoic acid, biotin, or inositol necessary for normal pigmentation was indicated from earlier work in this laboratory with rats¹ and dogs.² McGinnis, Norris, and Heuser³ likewise indicated the presence of an unidentified factor other than pantothenic acid in brewers' yeast which was necessary for normal feathering and pigmentation in growing Rhode Island Red chicks. Cure or prevention of the achromotrichia developed in rats fed sulfaguanidine or succinylsulfathiazole by administration of folic acid concentrates

was reported by Martin⁵ and by Wright and Welch.⁶ Similar results have been obtained in this laboratory using liver concentrates. It appeared therefore, that the active factor might be identified with the *L. casei* factor⁷ and vitamin B₁₂.⁸

Attempts have been made to develop techniques capable of assay for the unknown pigmentation factor. Chicks and dogs on highly purified diet and rats on purified diet with added succinylsulfathiazole have been used. Most consistent results have been obtained with growing chicks. In studying the role of the *L. casei* factor in hair and feather pigmentation, concentrates of the vitamin prepared by one of us (F.C.M.) and finally the

¹ Frost, D. V., Moore, R., and Dann, F. P., *Proc. Soc. Exp. Biol. and Med.*, 1941, **46**, 507.

² Dann, F. P., Moore, R., and Frost, D. V., *End. Proc. Soc. Exp. Biol. and Med.*, 1942, **1**, 107.

³ Frost, D. V., and Dann, F. P., *J. Nutrition*, 1944, **27**, 355.

⁴ McGinnis, J., Norris, I. C., and Heuser, G. F., *J. Biol. Chem.*, 1942, **145**, 341.

⁵ Martin, G., *Proc. Soc. Exp. Biol. and Med.*, 1942, **51**, 353.

⁶ Wright, R. D., and Welch, A. D., *J. Nutrition*, 1944, **27**, 55.

⁷ Phaff, J. J., et al., *Science*, 1943, **97**, 404.

⁸ Angier, R. B., et al., *Science*, 1945, **102**, 227.

TABLE I
 Composition of Basal Chick Diet

	g/100 g		mg/100 g
Sucrose	58.0	Thiamine	0.30
Casein S M A (vitamin free)	18.0	Riboflavin	0.40
Gelatin	10.0	Pyridoxine	0.40
Salts (O & M)	5.0	d(+)-Ca pantothenate	3.30
CaHPO ₄ · 2H ₂ O	1.0	Nicotinic acid	10.00
MnSO ₄ · 2H ₂ O	0.04	Mixed tocopherols	1.25
Corn oil	5.0	2 Methyl 1,4 naphthoquinone	0.44
Holiver oil	0.03	Biotin	0.036
Cystine	0.3	ZnCl ₂	1.25
Choline	0.15	CuSO ₄ · 5H ₂ O	1.50
Inositol	0.1		

pure synthetic substance were used*. Liver concentrates used in these experiments were first standardized against a liver fraction of known vitamin B₆ content†. The standardized liver fraction was later assayed against the synthetic *L. casei* factor. Standardizations against pure vitamin B₆ and synthetic *L. casei* factor gave the same values with both *L. casei* and *S. faecalis* R. The active material in our concentrates is hereafter referred to as *L. casei* factor.

The basal diet was designed to supply in adequate amount all dietary factors which had previously been associated with chromatrichial activity in various species, excepting only *p*-aminobenzoic acid. Particular regard was paid to the adequacy of copper,^{9, 10, 11} zinc,¹² pantothenic acid,^{13, 14, 15} biotin,¹⁶ and cystine.¹⁷ The chromatrichial activity originally reported for *p*-aminobenzoic¹⁸ acid in rats has not been verified.^{19, 15, 2} For this reason it was decided not to include it in the diet at the present stage of investigation.

Experimental. Day-old Black Leghorn chicks were fed a basal diet as shown in

Table I. In preliminary experiments, concentrates of the *L. casei* factor from liver were used to determine the levels needed for growth. Groups of 10 chicks were used and observations were recorded weekly. It was determined that a level of 400 µg per 100 g of diet gave good growth, feathering, and pigmentation over a period of 12 weeks. Growth was about 10% greater for control chicks receiving 10% liver fraction B (Wilson), but there was no observed difference in feathering and pigmentation. Chicks which received the *L. casei* factor concentrate for only the first 2 weeks grew at a decreasing rate and finally began to lose weight after 7 weeks, however, no failure in pigmentation or feathering was observed in this group even after 10 weeks.

Preliminary experiments were also conducted in which *L. casei* factor concentrates from liver were given 5 times weekly by injection in amounts calculated to supply 1, 2.5, 5, 10, and 20 µg of the factor per day. In this run the fullness of feathering was proportional to the level of *L. casei* factor up to the 10 µg

* We wish to thank Dr. E. L. R. Stokstad of Lederle Laboratories for supplying us the synthetic *L. casei* factor used in these studies.

† This liver fraction was standardized against crystalline vitamin B₆ by Prof. W. H. Peterson at the University of Wisconsin.

⁹ Gortel, F. J., *Nature*, 1935, **136**, 185.

¹⁰ Keil, H. L., and Nelson, V. E., *J. Biol. Chem.*, 1931, **93**, 49.

¹¹ Free, A. H., *Proc. Soc. Exp. Biol. and Med.*, 1940, **44**, 371.

¹² Stirn, E. E., Elvehjem, C. A., and Hart, E. B., *J. Biol. Chem.*, 1935, **109**, 347.

¹³ Gyorgy, P., and Polng, C. E., *Science*, 1940, **92**, 202.

¹⁴ Unna, K., Richards, G. V., and Sampson, W. L., *J. Nutrition*, 1941, **22**, 553.

¹⁵ Henderson, L. M., McIntire, J. M., Waisman, H. A., and Elvehjem, C. A., *J. Nutrition*, 1942, **23**, 47.

¹⁶ Gyorgy, P., and Polng, C. E., *Proc. Soc. Exp. Biol. and Med.*, 1940, **45**, 773.

¹⁷ Pavek, P., and Baum, H. M., *Proc. Soc. Exp. Biol. and Med.*, 1941, **47**, 271.

¹⁸ Anschuetz, S., *Science*, 1941, **93**, 164.

¹⁹ Emerson, G., *Proc. Soc. Exp. Biol. and Med.*, 1941, **47**, 448.

TABLE II

Growth, Feathering, and Pigmentation of Chicks Receiving Various Levels of Synthetic *L casei* Factor by Injection Comparison with *L casei* Factor Concentrate from Liver and with 10% Brewer's Yeast Supplement

Weeks	Synthetic <i>L casei</i> factor 1 μ g				Synthetic <i>L casei</i> factor 2.5 μ g				Synthetic <i>L casei</i> factor 5 μ g			
	Wt, g	Feathering	Pigmen- tation %		Wt, g	Feathering	Pigmen- tation %		Wt, g	Feathering	Pigmen- tation %	
1	44				46				46			
2	75				80				84			
3	106				127				146			
4	119				164				211			
5	132	Very poor	0		193	Poor	10		269	Subnormal	25	
6	153	" "	0		234	" "	10		310	" "	40	
7	157	" "	0		242	" "	40		369	" "	40	
8	176	" "	0		291	" "	40		436	" "	60	
9	172*	" "	10		308†	" "	50		459	" "	60	
Weeks	Synthetic <i>L casei</i> factor 10 μ g				<i>L casei</i> factor concentrate 10 μ g				10% brewer's yeast			
	Wt, g	Feathering	Pigmen- tation %		Wt, g	Feathering	Pigmen- tation %		Wt, g	Feathering	Pigmen- tation %	
1	44				45				45			
2	78				80				81			
3	130				135				147			
4	195				207				230			
5	264	Normal	100		275	Normal	90		300	Normal	100	
6	346	" "	100		348	" "	90		358	" "	100	
7	374	" "	100		409	" "	90		458	" "	100	
8	477	" "	100		495	" "	90		594	" "	100	
9	548	" "	100		544	" "	90		666	" "	100	

* Only 3 chicks survived

† Only 4 chicks survived

level where it was normal. Depigmentation was marked at the 5 and 10 μ g levels. At the lower levels, the feathers were poorly developed and the chicks had a ragged and scrawny appearance. Growth was in proportion to the level of *L casei* factor given.

When synthetic *L casei* factor was made available to us for research purposes, the above experiment was repeated at injection levels calculated to supply 1, 2.5, 5, and 10 μ g daily of the crystalline material. A control group with 10% brewers' yeast supplement added to the basal diet was run for comparison. The results as to growth, feathering, and pigmentation were essentially similar to those above except that the 10 μ g level of synthetic *L casei* factor appeared adequate for normal pigmentation, whereas the concentrate at this level had not proved adequate.

The results with synthetic *L casei* factor are shown in Table II. Photographs of repre-

sentative chicks from the various groups are shown in Fig. 1.

At the 1 and 2.5 μ g levels, feathering was so poor that true depigmentation was difficult to discern, although the chicks gave the general appearance of grayness. At the 5 μ g level, chicks showed the most definite depigmentation. The plumage of this group was somewhat sparse, but was full enough so that depigmentation was readily apparent. Both feathering and pigmentation were about equal for the group receiving 10 μ g *L casei* factor by injection and the control group fed yeast supplement.

Depigmentation became most pronounced in the proximal portions after the feathers were fairly well developed. The wing and tail feathers were most affected, but nearly all of the feathers were somewhat affected. Depigmentation was not discernible as quickly in the lower level groups as it was at the 5 μ g level, probably because feather growth was

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Salts (O & M)	5.0	d(+) C ₁ pantothenate	3.30
CaHPO ₄ 2H ₂ O	1.0	Nicotinic acid	10.00
MnSO ₄ 2H ₂ O	0.04	Mixed tocopherols	1.25
Corn oil	5.0	2 Methyl 1,4 naphthoquinone	0.44
Hatchery oil	0.03	Biotin	0.036
Cystine	0.3	ZnCl ₂	1.25
Gholme	0.15	CuSO ₄ 5H ₂ O	1.50
Inositol	0.1		

pure synthetic substance were used*. Liver concentrates used in these experiments were first standardized against a liver fraction of known vitamin B₆ content†. The standardized liver fraction was later assayed against the synthetic *L. casei* factor. Standardizations against pure vitamin B₆ and synthetic *L. casei* factor gave the same values with both *L. casei* and *S. faecalis* R. The active material in our concentrates is hereafter referred to as *L. casei* factor.

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¹³ Gyorgy, P., and Poling, C. E., *Science*, 1940, **92**, 202.

¹⁴ Umrigar, K., Richards, G. V., and Simpson, W. L., *J. Nutrition*, 1941, **22**, 553.

¹⁵ Henderson, L. M., McIntire, J. M., Waismann, H. A., and Elvehjem, C. A., *J. Nutrition*, 1942, **23**, 47.

¹⁶ Gyorgy, P., and Poling, C. E., *Proc. Soc. Exp. Biol. and Med.*, 1940, **45**, 773.

¹⁷ Pravek, P., and Baum, H. M., *Proc. Soc. Exp. Biol. and Med.*, 1941, **47**, 271.

¹⁸ Ausbrucher, S., *Science*, 1941, **93**, 164.

¹⁹ Emerson, G., *Proc. Soc. Exp. Biol. and Med.*, 1941, **47**, 448.

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¹⁰ Keil, H. L., and Nelson, V. E., *J. Biol. Chem.*, 1931, **93**, 49.

¹¹ Free, A. H., *Proc. Soc. Exp. Biol. and Med.*, 1940, **44**, 371.

¹² Stern, D. E., Elvehjem, C. A., and Hart, E. B., *J. Biol. Chem.*, 1935, **109**, 347.

at a level which proved adequate in the case of liver concentrates, based on *S faecalis* R assay. Thus it may prove true that the *L casei* assay is a better indication of activity for higher animals.

The importance of pantothenic acid in normal pigmentation has been adequately emphasized. The interesting observation has been further made⁶ that the storage and utilization of pantothenic acid in succinylsulfathiazole-fed rats is favorably influenced by the simultaneous administration of biotin and folic acid-rich concentrates. Early work in this laboratory¹ indicated that low levels, *i.e.*, 40-60 μ g of pantothenic acid as it occurred in natural products, were much more effective in prevention of graying in growing rats than levels up to 200 μ g per day of d(-)-calcium pantothenate. The inference was that other factors were acting synergistically with pantothenic acid. This idea seems to have support in much of the work to date, and it appears probable that the metabolic functions of the *L casei* factor and pantothenic acid are closely interdependent.

In line with the above reasoning it may now become possible to reconcile the discrepancies between different laboratories regarding the adequacy of pantothenate alone to cure or prevent achromotrichia in rats. The adequacy of given levels of pantothenate for growth and pigmentation may to some extent be governed by the level of *L casei* factor in the diet, or the rate of intestinal synthesis of this factor. Unna and Richards²² observed that the requirement of pantothenate for rats decreases from 100 μ g per day at 3 weeks of age to 25 μ g per day at 10 weeks of age. Furthermore, rats started on 25-50 μ g per day of pantothenate became gray but showed gradual

restoration of the black color of the fur as they approached maturity. These observations were confirmed by Henderson *et al.*¹⁵ On the other hand, Williams²³ reported the inefficacy of even high levels of pantothenate to cure graying uniformly, as was also observed in this laboratory.¹ Further experiments are indicated in which the *L casei* factor is supplied together with pantothenate.

The needs of the chick for the *L casei* factor are apparently not complicated by intestinal synthesis to the same extent as in the rat. For the latter reason, chicks may be considered preferable to other species in studies dealing with the effect of diet on normal pigmentation processes.

It is significant to note that Willher²⁴ has elaborated on the common role of melanophores in bringing about the pigmentation of hair or feathers in different species and has further postulated that a genetic lethal factor in the melanophore may be the primary cause of graying in man.

Summary Black Leghorn chicks were raised on a basal diet designed to contain the previously recognized chromotrichial factors, except the *L casei* factor. Synthetic *L casei* factor injected 5 times weekly at a level calculated to supply 10 μ g per day showed feathering and pigmentation comparable to that of chicks receiving 10% brewers' yeast supplement. Growth was somewhat better on the latter. At a level of 5 μ g, feathering was fairly good and depigmentation was marked. At 1 and 2.5 μ g levels, feathering was very poor. Growth and feathering were in proportion to the level of *L casei* factor used.

Acknowledgment is made to Mr. Albin Junnila for technical care of the animals.

²² Unna, K., and Richards, G. V., *J. Nutrition*, 1942, **23**, 545.

²³ Williams, R. R., *Science*, 1940, **92**, 561.

²⁴ Willher, B. H., *Ann. Surgery*, 1942, **116**, 598.

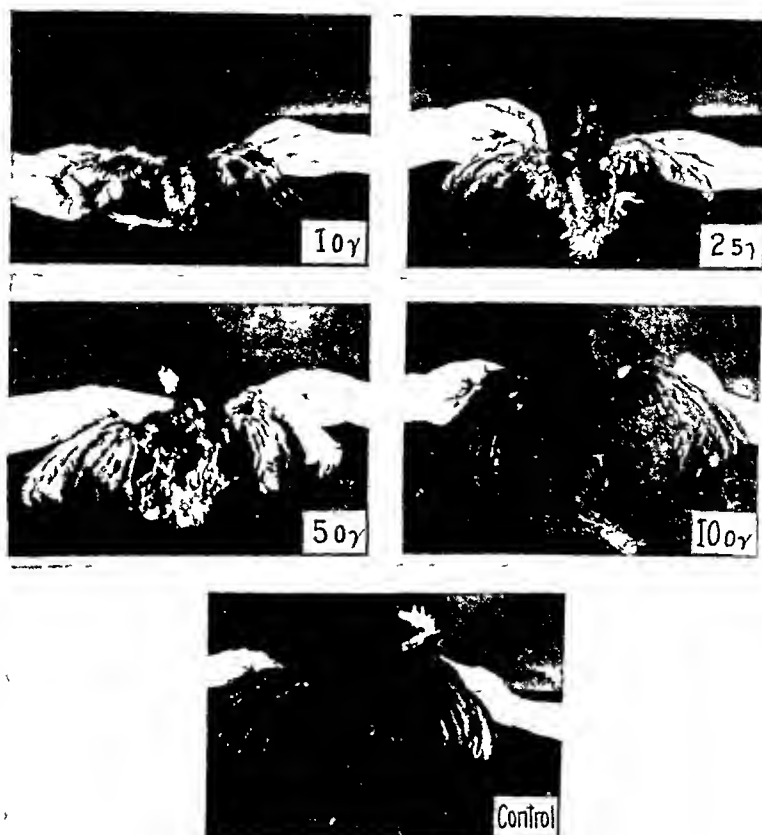


FIG 1

Chicks at nine weeks of age representing the groups which received 1, 2.5, 5, or 10 μ g daily of synthetic *L casci* factor by injection or 10% brewer's yeast added to the basal diet

slower and less complete at the lower levels

Discussion A primary need for the *L casci* factor in the chick would appear to be for feather development. When the need for feather formation is just satisfied, the needs for pigmentation are still not met. Depigmentation then becomes most apparent in the rapidly growing feathers. A still higher level is needed to satisfy both needs completely.

Campbell, Brown, and Emmett²⁰ have indicated that a level of 20 μ g daily of crystalline vitamin B₆ isolated from yeast, given either orally or parenterally to growing chicks, produces normal growth, feathering, and blood values. The present work indicates that a level of about 10 μ g of synthetic *L casci* factor

is adequate for normal feathering and gives good growth. It appears likely that a somewhat higher level would have given growth comparable to that obtained with 10% brewers' yeast supplement. Evidence for the existence of separate factors required for feathering and growth has been presented by Briggs, Luckey, Elvehjem, and Hart²¹. The multiplicity of forms in which the *L casci* factor apparently occurs in nature requires clarification with regard to this possibility.

In separate experiments we have used concentrates of a fermentation product highly active for *S faecalis* R, but only slightly active for *L casci*. These concentrates gave very poor growth and feathering when used

²⁰ Campbell, C. J., Brown, R. A., and Emmett, A. D., *J. Biol. Chem.*, 1944, **154**, 721.

²¹ Briggs, G. M., Luckey, T. D., Elvehjem, C. A., and Hart, E. B., *J. Biol. Chem.*, 1944, **153**, 423.

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²³ Williams, R. R., *Science*, 1940, **92**, 561.

²⁴ Willier, B. H., *Ann. Surgery*, 1942, **116**, 598.

Effect of Radon-Containing Ointment on Cutaneous Lymph Flow and Minute Vessels of Skin

J Q GRIFFITH, JR,* E P PENDERGRASS, C R PERRYMAN, AND J HOOKER

From the Robinette Foundation and the Department of Radiology, Hospital of the University of Pennsylvania

It had been the clinical experience of one of us (E P P) that "Alphatron" Radon Ointment[†] was of value in the treatment of ulcers resulting from previous excessive irradiation. The study here reported is an attempt to explain this effect. For clarity of presentation, the evidence will be presented in the following order: (1) Experiments suggesting that decreased lymphatic absorption, and presumably therefore some degree of lymphatic block, is present following the subsidence of the acute stage of post-irradiation reaction. (2) Experiments indicating that there is hyperemia of the active type in man and in animals following the use of Radon ointment. (3) Experiments showing in man and in normal animals there is increased lymphatic flow in the cutaneous area treated with Radon ointment. (4) Experiments in animals indicating that the active hyperemia and increased cutaneous lymphatic flow in animals is not associated with increased permeability of the capillaries to the colloidal dye Congo red, whereas such increase in permeability does occur following excessive irradiation by x-rays. (5) Result of the application of Radon ointment to cutaneous areas in men in whom local lymphatic block was presumed to have occurred following the injection of penicillin.

The normal human subjects were men between the ages of 22 and 41, the abnormal subjects, who received the penicillin, were men between the ages of 30 and 50 who were being treated for subacute bacterial endocarditis. The animals used were white rats, of either sex, some normal and others,

where stated, having been previously treated with roentgen irradiation totaling 2385 r, given in a single dose. Radon ointment in all cases contained 500 electrostatic units per cubic centimeter, was applied in a film approximately 1 mm thick, covered with oil silk fastened down at the edges by adhesive tape, and left in place for 4 hours when it was removed by gentle wiping with cotton. Control areas were treated similarly but using anhydrous lanolin instead of Radon. Studies were made 24 hours after the application of the Radon ointment, unless otherwise expressly stated.

1 Lymphatic absorption following x-ray irradiation. Lymphatic flow in the skin may be measured either by (a) injecting a colored colloidal dye as patent blue¹ into the skin and measuring the speed and extent of streamer formation, or (b) measuring the amount of time required for complete absorption of a standard amount of dye so injected. The first method, measuring streamers, is well adapted to show increased lymphatic flow, but normal flow is so slow that decreased flow cannot be so demonstrated, the second method is better adapted to demonstrating diminished flow.

Sixteen rats were used that 3 months previously had received roentgen therapy to one leg. Patent blue, 0.04 cc in amount, was injected intracutaneously into the dorsum of both feet, one irradiated, and the other a control. After 24 hours, the dye had completely disappeared from both feet in 6 rats, while in the other 10 it was present on the irradiated but not on the control side.

2 Hyperemia following application of Radon ointment. The antecubital area of the forearm was examined under the capillary

* Atwater Kent Fellow in Medicine

[†] Obtained from the Canadian Radium and Uranium Corporation, 630 Fifth Ave., New York City, which also provided funds to defray part of the expense of the study.

microscope² before and 24 hours after the application of Radon ointment, and compared with the other arm prepared as a control. The appearance of the control arm was the same on both days. The treated arm showed an increase in capillary loops of approximately 100% in 7 subjects, along with some increase in subpapillary venous plexus in 5 of the subjects. The color was bright red, not cyanotic, but otherwise it could not be told whether the hyperemia was active or passive. However, the dorsum of the foot of 6 rats, similarly treated and with the opposite leg as a control indicated quite clearly that the hyperemia was active. In this area, the rat does not open and close cutaneous vessels as does man in the forearm, but usually only about 30% of the vessels show flow. This was true of the control leg on both days and of the treated leg before treatment, but after treatment about 90% of the vessels showed flow, a result very comparable to that obtained 4 or 5 days after lumbar sympathectomy.

3 Lymphatic flow following application of Radon ointment. Radon ointment was applied in the usual way for 4 hours to the antecubital area of one arm in 8 normal men and 12 hours later 0.04 cc of patent blue was injected intracutaneously into the treated area and into a corresponding area of the control arm. Streamer formation was followed at 5 minute intervals for 15 minutes, and found to be significantly increased on the treated side in 6 cases and not significantly different in 2 cases. Disappearance time was measured in 5 cases, and ranged from 8 to 10 hours, averaging 9 hours, on the treated side, and 24 to 48 hours, averaging 36 hours, on the control side.

Under sodium phenobarbital anesthesia lasting about 24 hours, 14 rats were treated with Radon ointment to one foot using the same dosage and time factors as for the human subjects. However immediately after the Radon and control dressings were removed, both feet were injected intracutaneously with an equal amount of patent blue. Twenty hours later it was noted that dye

was present in the control foot but not in the treated foot of 7 animals, and had cleared entirely from both feet in the remaining 7 animals. In no case was dye present in the treated leg after the control leg had cleared.

4 Permeability of capillaries to Congo red after the application of Radon ointment. Under sodium phenobarbital anesthesia, Radon ointment was applied to one foot of 8 normal rats, and immediately thereafter 0.3 cc of a 5% solution of the colloidal dye Congo red was injected into a jugular vein. The animals were observed at intervals for 24 hours. At no time was the treated leg any pinker than the control leg or the rest of the body. By contrast, Congo red was injected similarly into 7 animals that had received 2385 r of x-ray irradiation to one leg one month previously. Within 5 minutes the treated leg had become very red as compared with the control leg and other parts of the body.

5 Four men were available in the medical ward who had received many injections of penicillin. One man in particular had received hundreds of injections over a period of 8 months, with so much resulting discomfort that he was about to refuse further therapy. It seemed likely that the local swollen areas represented areas of local lymphedema due to acute partial lymphatic block from colloidal and particulate matter in the penicillin. Improvement was striking in 12 hours. An arm that had been held rigidly because of a painful swelling near the scapula was freely moveable and the swelling was greatly decreased in size and not tender. Previous therapy, over a period of more than 3 months with heat, baking light et cetera had been completely ineffective in relieving the pain and swelling. In all, 12 applications of Radon ointment were given to 4 patients over areas where a painful swelling had appeared following an injection of penicillin. Six control applications were given using either the inactive ointment or Radon ointment from a tube at least 2 weeks old. In every case the control applications were recognized by the patient as being ineffective, and the treatments as effective.

Summary and Conclusions Evidence is

² Griffith, J. Q., Jr., Roberts, E., and Corbit, H. O., *Am Heart J*, 1941, 21, 47.

presented suggesting that the effect of Radon ointment applied to the skin of men and animals is (1) To produce active hyperemia (2) To increase lymph formation and lymph flow without appreciably increasing capillary permeability to colloids (3) It is suggested that the therapeutic effect of Radon ointment may be apparent in these cases where lymph flow is diminished, per-

haps due to partial block. Evidence is offered suggesting that lymph flow is diminished in a limb that has, in the past, been exposed to excessive x-ray irradiation. Also, the result of treating acute lesions thought to represent partial lymphatic block following penicillin injections is described, with results that are suggestively good although the series is too small for absolute certainty.

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Effects of Glutathione and Thioglycolic Acid on Acid-Production by *Lactobacillus arabinosus* in Presence of Atabrine

H F FRASER, FILADELFO IRREVERRE, AND MARIE M GRENAN
(Introduced by M Pittman)

From the National Institute of Health, Bethesda, Md

The action of atabrine as a competitive inhibitor of certain enzyme systems has been investigated by Wright and Sabine¹ and Haas.² The influence of different types of diets upon the toxicity of atabrine for various laboratory animals has been studied by several groups of workers, and a recent article by Siegel, Mushett and Emerson³ reviews the contributions in this field.

Silverman and Evans⁴ reported on the effects of spermine, spermidine, and other polyamines on the growth inhibition of *Escherichia coli* and certain soil organisms by atabrine. These organisms are capable of growth on an inorganic salts-glucose medium and atabrine bacteriostasis is relieved by spermidine. On the other hand, they observed that atabrine bacteriostasis produced in soil organisms with complex nutrient requirements is not relieved by spermidine and related polyamines.

The purpose of this paper is to present data respecting the response of the *Lactobacillus arabinosus* (an organism with relatively

complex nutrient requirements) to atabrine with and without the addition of glutathione and thioglycolic acid to the medium.

Materials and Methods A strain of *Lactobacillus arabinosus* 17-5 was used. This was supplied by Dr Jerald G Wooley of the Division of Physiology, National Institute of Health. The procedure for growth of stock cultures and preparation of culture for inoculation was that of the U S Pharmacopeia,⁵ using the technics employed by the Food and Drug Administration. The basal medium and stock solutions were that of the U S Pharmacopeia for nicotinic acid assays⁵ except that 0.25 g of bacto-dextrose, 0.20 g of sodium acetate and 0.5 µg of nicotinic acid were added to each tube, i.e., 10 ml final volume.

A distilled water solution of C P atabrine dihydrochloride dihydrate ($2.0 \times 10^{-3} M$) was added to the tubes in varying amounts in such a range that minimum and maximum bacteriostatic effects were demonstrated. Comparable tubes were set up which contained distilled water solutions of glutathione, Merck, or thioglycolic acid practical, Eastman, in a final concentration of 1.6×10^{-3} molar. In these experiments the pH of the medium was not altered by the addition of

¹ Wright, C I, and Sabine, T C, *J Biol Chem*, 1944, **155**, 315.

² Haas, E, *J Biol Chem*, 1944, **155**, 321.

³ Siegel, H, Mushett, C W, and Emerson, G A, *Proc Soc Exp Biol and Med*, 1945, **58**, 157.

⁴ Silverman, M, and Evans, E A, *J Biol Chem*, 1944, **154**, 521.

⁵ *U S Pharm*, XII First Round Supplement, 1943.

TABLE I
Percent Inhibition of Acid Production by *Lactobacillus arabinosus* in the Presence of Atabrine Alone, Atabrine + Glutathione, and Atabrine + Thioglycolic Acid

Molar concentration of atabrine in tube	% inhibition of acid production		
	Atabrine alone %	Atabrine + glutathione %	Atabrine + thioglycolic acid %
0	0	0	0
1.0×10^{-4}	24	27	6
1.2×10^{-4}	42	28	14
1.6×10^{-4}	91	50	29
2.0×10^{-4}	100	85	56
2.4×10^{-4}	98	98	90

glutathione and thioglycolic acid. The amount of bacterial growth after autoclaving, inoculation and incubation for 66 hours was measured by titrating each tube for acid production with 0.1 N NaOH. A blank was always run with all the ingredients but no inoculation; the amount of 0.1 N NaOH required for this blank was routinely subtracted from the titration values of the comparable inoculated tube. One hundred percent growth was the value obtained in the control tube which contained no atabrine and was inoculated. The percent inhibition of growth induced by atabrine was computed by comparing the acid production by titration under control conditions, when no atabrine was added, with acid production under experimental conditions when atabrine was added. When the effect of glutathione and thioglycolic acid was being tested the control tubes without atabrine contained an amount of glutathione or thioglycolic acid comparable to that put in the experimental tubes.

Results. The percent inhibition of acid production with atabrine alone, atabrine plus glutathione, and atabrine plus thioglycolic acid is shown in Table I. The data demonstrate that glutathione and thioglycolic acid are able to reverse the bacteriostatic effect of atabrine over a restricted range of concentration of the latter. For example, when the molar concentration of atabrine was 1.6×10^{-4} the percent inhibition of acid production was 91%, but with a similar concentration of atabrine plus glutathione the inhibition was 50%, and only 29% in the

case of atabrine plus thioglycolic acid. In the experiments carried out it was not possible to counteract the effects of the higher molar concentrations of atabrine by increasing the amounts of glutathione and thioglycolic acid. Lower concentrations of glutathione and thioglycolic acid than 1.6×10^{-3} were effective in counteracting atabrine but the results were not consistent.

In addition to the above compounds, which counteracted the effect of atabrine, several others were tried under comparable conditions with negative results, these included D-cystine, L-cystine, cysteine, DL-methionine, choline, nicotinic acid, riboflavin, and pyridoxine.

In the case of *E. coli* it has been shown^{6,4} that shifting the pH of the medium to values below 6.0 (which permit satisfactory growth of *E. coli*) is effective in reversing the action of atabrine. This was tried in the case of *Lactobacillus arabinosus* and a comparable effect was attained.

Summary. Under the conditions of these experiments glutathione and thioglycolic acid are capable of reversing the growth inhibiting effect of atabrine on the *Lactobacillus arabinosus*. Once the concentration of atabrine for complete inhibition had been significantly exceeded, no reversal of bacteriostasis was attained by increased concentration of glutathione and thioglycolic acid. Several additional compounds were tested under comparable conditions with negative results.

⁶ Browning, C. E., Gulbransen, R., and Keeney, E. L., *J. Path. and Bact.* 1919, **23**, 106.

presented suggesting that the effect of Radon ointment applied to the skin of men and animals is (1) To produce active hyperemia (2) To increase lymph formation and lymph flow without appreciably increasing capillary permeability to colloids (3) It is suggested that the therapeutic effect of Radon ointment may be apparent in these cases where lymph flow is diminished, per-

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⁴ Silverman, M, and Evans, E A, *J Biol Chem*, 1944, **154**, 521.

⁵ *U S Pharm*, XII First Bound Supplement, 1943.

TABLE I
Maternal Leucocytes During Lactation

Experimental diet	Day of lactation (No of rats)	Total* WBC	Lymphocytes and monocytes		PMN	
			%	Total*	%	Total*
Stock Diet I + lettuce	Dry 5 (10)	13,095 (8,000 21,000)	70% (5,200 14,175)	9,325	30% (1,710 4,945)	3,770
	" 21 (10)	12,375 (7,350 17,300)	72% (4,770 14,530)	9,035	28% (2,350 4,720)	3,340
	" 21 (10)					
Purified Diet 836	" 5 (7)	11,845 (6,150 17,400)	72% (4,670 10,265)	8,430	28% (1,480 7,135)	3,415
	" 21 (11)	5,205 (2,625 9,600)	83% (2,310 7,200)	4,195	17% (215 2,730)	1,010
	" 21 (11)					
Purified Diet 836 + 1 g brewers' yeast daily	" 5 (8)	12,850 (8,500 19,000)	69% (4,385 16,500)	9,025	31% (1,700 6,690)	3,825
	" 21 (9)	7,200 (3,400 12,350)	79% (3,400 10,375)	5,530	21% (0 4,405)	1,670
	" 21 (9)					
Purified Diet 836 + 1 cc liver extract daily	" 5 (8)	13,245 (10,800 17,000)	68% (6,780 13,040)	9,000	32% (1,860 7,515)	4,245
	" 21 (8)	8,005 (5,670 9,775)	74% (3,995 8,435)	5,950	26% (645 3,400)	2,055
	" 21 (8)					

* The range is given in parentheses below the average value

mm³) occurred in two-thirds of the group. Daily oral supplementation with 1 cc liver extract (Lederle 93% alcohol liver concentrate H-9381-B) resulted in some improvement but not in a return to the normal blood picture. 1 g brewers' yeast was somewhat less beneficial.

Preliminary studies were carried out with a folic acid concentrate (potency 5,000) kindly furnished by Dr R J Williams, University of Texas. Oral supplements of this concentrate, equivalent to 150 µg "folic acid" (i.e. 75 µg daily) were given during the 3-week period to the lactating mothers without beneficial effects on any of the criteria for lactation. While the effects of "folic acid" concentrates on the other criteria for lactation are questionable,^{3,6} there is no doubt concerning the curative effects of such concentrates or of crystalline vitamin Bc

(total dose 100 µg) on the leucopenia and granulocytopenia of non-lactating rats maintained on purified diets with sulfa drugs⁷ or even without their addition.⁸ Either considerably higher levels of "folic acid" or additional factors must be necessary for a normal leucocyte picture during the strain of lactation.

Summary Adult stock rats maintained on stock diets show no changes in the white blood cells during the 3-week lactation period. Maintenance on a purified diet during the same period results in a marked leucopenia and granulocytopenia by the twenty-first day. Daily supplements of brewers' yeast or liver extract result in improvement but not entire prevention of the blood dyscrasia.

⁷ Sebiell, W H, and Daft, F S, *Pub Health Rep*, 1943, **58**, 1542

⁸ Kornberg, A, Daft, F S, and Sebiell, W H, *Proc Soc Exp Biol and Med*, 1945, **58**, 46

⁶ Cerecedo, L R, and Vinson, L J, *Arch Biochem*, 1914, **5**, 469

Lactation Leucopenia in Rats on Purified Diets *

MARJORIE M NELSON,[†] FREEKE VAN NOUHUYS, AND HERBERT M EVANS*From the Institute of Experimental Biology, University of California, Berkeley, Calif*

Recently long-term experiments have shown that purified diets for rats are equivalent to stock diets for growth^{1,2} and reproduction² but not for lactation.^{1,2,3} The criteria used for lactation have been the percentage of young weaned, their average weight at weaning, and the loss in weight of the lactating mother during the lactation period. In comparison with the 60-70% weaning reported by other investigators^{1,3} working with purified diets, in this laboratory rats maintained throughout their entire life on a purified diet (836[†]) have weaned 90% of their young.² Since this is equivalent to the lactation performance of stock animals in our rat colony, this criterion has been eliminated from our work. By placing adult stock ani-

mals with their litters on the purified diet at parturition it is possible to test dietary factors in 3 weeks for their effects on the weaning weight of the young and the weight loss of the mother.² We now wish to report an additional criterion for testing the adequacy of purified diets, namely, the occurrence of leucopenia in the lactating mother during this short period.

Procedure Adult stock females with their litters were placed on the purified diet (836[†]) at parturition or were continued on the stock diet⁵ as controls. The litters were limited to 6 young, preferably 3 males and 3 females, which were weaned on the twenty-first day following parturition. Total and differential white blood counts were carried out by standard procedures at 2 different times, near the beginning of the lactation period (Day 5) and at the time of weaning (Day 21).

Results and Discussion Table I shows typical results for rats maintained on the stock diet or on the purified diet with or without supplements. There was no change in total WBC, lymphocytes and monocytes, or granulocytes during the lactation period in animals maintained on the stock diet whereas a marked leucopenia occurred by the twenty-first day in animals maintained on the purified diet (836). All animals receiving the purified diet (without any supplementation) showed a significant decrease in granular and non-granular leucocytes from their original level (Day 5) and the drop to pathological levels of granulocytes (*ie* granulocytopenia, less than 750 cells per

* Aided by grants from the Board of Research and from the Department of Agriculture of the University of California, and the Rockefeller Foundation, New York City. The following materials were generously contributed: crystalline B vitamins from Hoffmann-La Roche Company, Nutley, N J, Lederle Laboratories, Inc., Pearl River, N Y, and Merck and Company, Inc., Rahway, N J, alpha-tocopherol from Merck and Company, Inc., Rahway, N J, 93% alcohol liver concentrate (H 9381 B) from Lederle Laboratories, Inc., Pearl River, N Y.

[†] General Mills Fellow.

¹ Vinson, L J, and Cerecedo, L R, *Federation Proc*, 1943, **2**, 73, *Arch Biochem*, 1944, **3**, 389.

² Nelson, M M, and Evans, H M, in preparation.

³ Richardson, L R, and Hogan, A G, *Federation Proc*, 1945, **4**, 161.

⁴ Salts No 4 of Hegsted, D M, Mills, R C, Elvehjem, C A, and Hunt, E B, *J Biol Chem*, 1944, **138**, 459.

[†] Basis diet 836 consists of alcohol-extracted casein 24%, sucrose 64%, hydrogenated vegetable oil (Crisco) 8%, and salts⁴ 4%. Crystalline B vitamins are added per kilogram diet: thiamine HCl 5 mg, pyridoxine HCl 5 mg, riboflavin 10 mg, p-aminobenzoic acid 10 mg, nicotinic acid 20 mg, calcium pantothenate 50 mg, inositol 400 mg, and

choline chloride 1 g. One cc of a fat-soluble vitamin mixture containing 6 mg alpha-tocopherol, 115 cholecalciferol units vitamin D, 800 U.S.P. units vitamin A, and 650 mg corn oil (Mazola) is given weekly to each litter.

⁵ Wainwright, W W, and Nelson, M M, *Am J Orthodontics and Oral Surgery*, 1945, **31**, 406.

TABLE I
Murine Typhus

Guinea pig No	Incubation (days)	Fever (days)	Serotal reaction	Complement fixation titer		
				Epidemic	Murine	Rocky Mountain spotted fever
3322	3	7	+	1/10	1/320	0
3328	3	7	+	1/20	1/160	0
3329	3	7	+	1/20	1/320	0
3325	4	6	+	1/10	1/160	0
3326	3	6	+	1/20	1/320	0
3330	3	6	0	1/20	1/320	0
3338	3	6	+	1/80	1/320	0
3347	4	6	0	1/80	1/640	0
3317	3	5	+	1/80	1/320	0
3319	3	5	+	0	1/160	0
3321	3	5	+	1/20	1/160	0
3331	3	5	+	1/10	1/160	0
3332	3	5	+	1/10	1/160	0
3334	3	5	+	1/20	1/320	0
3337	3	5	+	1/10	1/80	0
3341	4	5	0	1/40	1/320	0
3323	4	4	+	1/20	1/320	0
3335	5	4	+	1/80	1/640	0
3344	5	4	+	1/20	1/160	0
3350	3	4	+	1/10	1/160	0
3308	6	3	0	1/40	1/320	0
3311	3	3	0	1/10	1/320	0
3316	5	3	+	1/80	1/320	0
3318	4	3	+	0	1/160	0
3320	4	3	+	1/20	1/320	0
3324	4	3	+	1/20	1/320	0
3327	5	3	+	1/20	1/320	0
3349	5	3	+	0	1/160	0
3355	3	3	+	1/40	1/320	0
3307	5	2	0	1/10	1/160	0
3309	5	2	0	1/40	1/320	0
3313	4	2	+	1/40	1/640	0
3333	7	2	0	1/20	1/320	0
3339	4	2	0	1/20	1/320	0
3345	5	2	+	1/20	1/320	0
3352	5	2	+	1/80	1/640	0
3306	5	1	+	1/20	1/160	0
3314	6	1	+	1/40	1/320	0
3346	5	1	+	1/20	1/320	0
3354	5	1	+	1/20	1/320	0
3342	0	0	0	1/20	1/320	0
3348	0	0	0	1/10	1/160	0

fever and vice versa ^{1 2}

During the past 3 years, this laboratory has employed the specific complement fixation test on specimens of convalescent guinea pig serum to differentiate epidemic and murine typhus and Rocky Mountain spotted fever. This paper will deal with the results obtained in an experimental study of infections in guinea pigs produced with strains of these 3 agents.

¹ Cisternici, M. R., and Silvi, R., *J. Immunol.*, 1941, **42**, 1.

² Parker, R. R., *Public Health Rep.*, 1943, **58**, 721.

Experimental Murine Typhus A series of 50 male guinea pigs (400-500 g) was bled prior to inoculation in order to obtain baseline specimens. They were then inoculated intraperitoneally at the same time with the same pooled suspension of 1 cc of tunica vaginalis exudate—Wilmington strain (Both tunica vaginalis and testicular exudate are suspended in 20 cc of physiological saline). Forty-two of the 50 guinea pigs survived the entire experiment, the others dying of intercurrent infections.

Forty guinea pigs developed fever while 2

Identification of Rickettsial Agents Isolated in Guinea Pigs by Means of Specific Complement Fixation

HARRY PLOTZ,* K. WERTMAN, AND B. L. BENNETT*

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The isolation of the agents of rickettsial diseases of man are usually made in guinea pigs (scrub typhus is an exception, since the white mouse is more susceptible). Once isolated the agent is identified by its capacity to induce a febrile and pathologic response in guinea pigs (scrotal swelling or necrosis, enlarged spleen, etc.) or the absence of these reactions in other species of animals, followed by the demonstration of a reciprocal cross immunity with known rickettsial strains. This procedure is not only time-consuming and expensive but presents certain difficulties because strains of the agent of a given disease may induce reactions in guinea pigs of different degree and intensity. The problem of strain identification, based on animal reactions alone, can therefore, present certain difficulties, some of which are discussed here.

When human blood, from a case of epidemic typhus, is inoculated intraperitoneally into male guinea pigs (400-500 g), the incubation period may vary from 6 to 20 days followed by a febrile period of variable duration. When brain suspensions from these animals are passaged to other male guinea pigs the incubation period is shortened to 6 or 7 days but may vary from 4 to 14 days. (Data based on 54 separate isolations).†

While scrotal swelling is not frequent in epidemic typhus, this reaction may occasionally occur. Since epidemic and murine typhus confer a reciprocal cross immunity in guinea pigs further identification of the agent is made in white rats, in which animal the epidemic disease cannot be maintained by

serial passage, while this is possible with murine typhus. Since fever is an important expression of the experimental disease, the interpretation of a reaction can be confused when fever is caused by conditions other than typhus. Furthermore, there are a certain number of guinea pigs that develop an inapparent disease, which is characterized by the absence of a febrile reaction.

When infectious human blood from a case of murine typhus is inoculated intraperitoneally into male guinea pigs (400-500 g), the incubation period can vary from 4 to 14 days followed by a febrile period of variable duration. When scrotal exudate from these animals is passaged to other guinea pigs, the incubation period is usually shortened to about 4 or 5 days with a variation of from 2 to 10 days. This is then followed by a febrile period of variable duration.† While scrotal swelling is usually present in large guinea pigs and Neil-Mooser cells are readily demonstrated, this reaction may be absent in animals even though they develop a febrile reaction. There are other guinea pigs that develop an inapparent disease, with no febrile or scrotal reaction whatsoever.

The type of reaction induced in guinea pigs with the agent of Rocky Mountain spotted fever depends upon whether a mild or virulent strain is isolated. The period of incubation with the former is usually longer, furthermore, no scrotal necrosis occurs and the guinea pigs usually survive. With the virulent strain, the period of incubation is shorter, scrotal necrosis develops and death occurs in the majority of the animals inoculated. Identification of the spotted fever agent by cross immunity tests may cause some difficulty since a partial immunity to Rocky Mountain spotted fever is found in guinea pigs convalescent from typhus.

* Member, United States of America Typhus Commission

† Same technique used throughout. Details to be published.

TABLE II
Epidemic Typhus

Guinea pig No	Incubation (days)	Fever (days)	Complement fixation titer		
			Epidemic	Murine	Rocky Mountain spotted fever
764	6	10	1/320	1/10	0
765	5	9	1/320	0	0
771	5	9	1/320	0	0
981	5	9	1/640	1/20	0
759	8	8*	1/160	0	0
761	6	8*	1/320	1/10	0
768	5	8	1/160	0	0
994	6	8	1/160	0	0
736	8	7	1/320	0	0
738	6	7*	1/640	1/40	0
738	7	7	1/320	0	0
767	9	7	1/160	0	0
769	5	7	1/320	0	0
740	6	6	1/160	0	0
775	7	6	1/640	0	0
979	6	6*	1/320	0	0
980	5	6	1/320	0	0
984	7	6	1/320	0	0
739	7	5	1/320	0	0
757	12	5	1/80	0	0
770	10	5	1/160	0	0
742	9	4	1/320	1/10	0
743	7	4	1/640	0	0
772	10	4	1/160	0	0
773	9	4	1/160	0	0
987	9	4	1/320	0	0
990	10	4	1/160	1/20	0
991	6	4	1/160	0	0
766	5	3	1/160	1/20	0
760	6	3	1/640	1/20	0
977	19	2	1/640	0	0
985	7	1	1/80	0	0
986	15	1	0	0	0
737	0	0	0	0	0
741	0	0	0	0	0
763	0	0	0	0	0
983	0	0	0	0	0
988	0	0	0	0	0
989	0	0	1/160	0	0

* Also had serotral swelling

Mountain spotted fever antigen Guinea pig 986 that developed no complement fixing antibodies, had fever of 104.5° F for one day's duration on the 15th day after inoculation. When subsequently challenged, with an epidemic strain, this animal responded with a febrile reaction, furthermore the epidemic complement fixing antibodies then appeared in its serum. Thus the fever which was observed in this animal on the 15th day after primary inoculation was due to causes other than epidemic typhus. The other 31 members of the group were immune when challenged with epidemic typhus material.

There were 6 guinea pigs that did not develop a febrile reaction after the initial inoculation of infectious material (see No 737, 741, 763, 983, 988, and 987 in Table II). It is observed that one of these guinea pigs, No 989 developed specific epidemic complement fixing antibodies in convalescence. This animal proved to be immune to epidemic typhus when challenged subsequently. Therefore guinea pig No 989 had an inapparent infection as a result of the primary inoculation. In contrast the other 5 guinea pigs in this group were not immune when challenged with an epidemic strain. It is considered

showed no febrile reaction. The period of incubation varied from 3 to 7 days and the febrile period from 1 to 7 days. Eleven or 26% of the 42 guinea pigs did not develop a scrotal reaction and 2 of these likewise did not develop fever.

About 4 weeks after the initial inoculation, all of the guinea pigs were bled and complement fixation tests for epidemic and murine typhus and Rocky Mountain spotted fever were performed, using the specific purified rickettsial antigens already described^{3,4,5,6}. It had been shown that the soluble or "common" antigen gave cross fixation between epidemic and murine typhus and, hence, differentiation between these diseases could not be made when this material was present. Differentiation between these closely related diseases was possible, however, when washed rickettsial suspensions, free of soluble antigen, were used as antigen. Table I summarizes the results obtained.

All pre-inoculation specimens were negative in complement fixation tests with the epidemic, murine and Rocky Mountain spotted fever antigens. The convalescent specimens all gave positive reactions with the murine antigen, the titers varied between 1/80 to 1/640 with values of 1/320 or greater in more than half of the specimens. All but 3 of the sera also reacted with the epidemic antigen, nevertheless, the titers were always considerably lower than those obtained with the murine antigen. The serological response of guinea pigs 3307, 3309, 3311, 3330, 3333, 3339, 3341, 3342, 3347 and 3348, which developed a febrile reaction without scrotal swelling, was indistinguishable from that of guinea pigs which developed both fever and scrotal swelling. Guinea pigs 3342 and 3348 were of particular interest, for they had neither a febrile reaction nor scrotal swelling, yet they

also developed complement fixing antibodies. This serological response, together with the subsequent demonstration of an immunity to re-infection, indicated that these 2 guinea pigs had had an inapparent infection. There appears to be no correlation between the titers obtained and the severity of the experimental infection, as judged by the duration of the febrile period or the presence or absence of scrotal swelling. All specimens of serum were negative in tests with the Rocky Mountain spotted fever antigen.

One month after the temperatures returned to normal the guinea pigs were challenged by the intraperitoneal inoculation of 1 cc of a tunica vaginalis exudate suspension (Wilmington strain). All of the guinea pigs proved immune, while the controls reacted in the usual manner.

Epidemic Typhus. Fifty male guinea pigs (400-500 g) were bled for determination of pre-inoculation antibody levels and then injected intraperitoneally at the same time with 1 cc of a 10% pooled brain suspension (Breinl strain—guinea pigs sacrificed on the third febrile day). Thirty-nine of the 50 guinea pigs survived the entire experiment, the others dying of intercurrent infection. Thirty-three guinea pigs developed fever while 6 showed no febrile reaction. The period of incubation varied from 5 to 19 days followed by a febrile period of from 1 to 10 days. Four of the guinea pigs developed scrotal swelling. About 4 weeks after the initial inoculation all of the guinea pigs were bled and complement fixation tests for epidemic and murine typhus and Rocky Mountain spotted fever were performed on samples of serum. Table II summarizes the results obtained.

All pre-inoculation specimens gave negative results in complement fixation tests with the epidemic, murine or Rocky Mountain spotted fever antigens. Sera from 32 of the 33 guinea pigs that developed fever showed complement fixation titers of 1/80 to 1/640, the majority being 1/320 or higher, with the epidemic antigen. When cross fixation occurred with the murine antigen, the titers in these 8 instances were low, never exceeding 1/40. None of the specimens reacted with the Rocky

³ Plotz, H., and Wertman, K., *Science*, 1942, **95**, 441.

⁴ Plotz, H., *Science*, 1943, **97**, 20.

⁵ Plotz, H., Wertman, K., Bennett, B. L., Report to the Surgeon General, February 15, 1944, to be published.

⁶ Plotz, H., Wertman, K., and Reagin, R. L., *Bulletin of the U S Army Medical Department*, 1944, **79**, 40.

TABLE II
Epidemic Typhus

Guinea pig No	Incubation (days)	Fever (days)	Complement fixation titer		
			Epidemic	Murine	Rocky Mountain spotted fever
764	6	10	1/320	1/10	0
765	5	9	1/320	0	0
771	5	9	1/320	0	0
981	5	9	1/640	1/20	0
759	8	8*	1/160	0	0
761	6	8*	1/320	1/10	0
768	5	8	1/160	0	0
994	6	8	1/160	0	0
736	8	7	1/320	0	0
738	6	7*	1/640	1/40	0
758	7	7	1/320	0	0
767	9	7	1/160	0	0
769	5	7	1/320	0	0
740	6	6	1/160	0	0
775	7	6	1/640	0	0
979	6	6*	1/320	0	0
980	5	6	1/320	0	0
984	7	6	1/320	0	0
739	7	5	1/320	0	0
757	12	5	1/80	0	0
770	10	5	1/160	0	0
742	9	4	1/320	1/10	0
743	7	4	1/640	0	0
772	10	4	1/160	0	0
773	9	4	1/160	0	0
987	9	4	1/320	0	0
990	10	4	1/160	1/20	0
991	6	4	1/160	0	0
766	5	3	1/160	1/20	0
760	6	3	1/640	1/20	0
977	19	2	1/640	0	0
985	7	1	1/80	0	0
986	15	1	0	0	0
737	0	0	0	0	0
741	0	0	0	0	0
763	0	0	0	0	0
983	0	0	0	0	0
988	0	0	0	0	0
989	0	0	1/160	0	0

* Also had scrotal swelling

Mountain spotted fever antigen Guinea pig 986 that developed no complement fixing antibodies, had fever of 104.5° F for one day's duration on the 15th day after inoculation. When subsequently challenged, with an epidemic strain, this animal responded with a febrile reaction, furthermore, the epidemic complement fixing antibodies then appeared in its serum. Thus the fever which was observed in this animal on the 15th day after primary inoculation was due to causes other than epidemic typhus. The other 31 members of the group were immune when challenged with epidemic typhus material.

There were 6 guinea pigs that did not develop a febrile reaction after the initial inoculation of infectious material (see No 737, 741, 763, 983, 988, and 987 in Table II). It is observed that one of these guinea pigs, No 989, developed specific epidemic complement fixing antibodies in convalescence. This animal proved to be immune to epidemic typhus when challenged subsequently. Therefore, guinea pig No 989 had an inapparent infection as a result of the primary inoculation. In contrast, the other 5 guinea pigs in this group were not immune when challenged with an epidemic strain. It is considered

TABLE III
Rocky Mountain Spotted Fever

Guinea pig No	Incubation (days)	Fever (days)	Complement fixation titer		
			Epidemic	Murine	Rocky Mountain spotted fever
294	3	9	0	0	1/320
280	3	8	0	0	1/320
323	2	8	0	0	1/320
290	3	7	0	0	1/640
292	4	7	0	0	1/320
276	3	7	0	0	1/640
260	4	7	0	0	1/320
286	4	7	0	0	1/320
288	4	7	0	0	1/320
190	6	7	0	0	1/320
191	2	6	0	0	1/320
284	5	5	0	0	1/320
189	6	2	0	0	1/640

that these animals represent "misses" in that the inoculated infectious material induced neither fever, an antibody response nor immunity.

In another series of 50 guinea pigs infected with different strains of epidemic typhus, 2 guinea pigs were observed which developed no febrile reaction. Convalescent specimens of serum from both of these guinea pigs showed the presence of epidemic complement fixing antibodies and the animals proved immune on challenge. The other 48 animals in this series displayed fever after primary inoculation, developed complement fixing antibodies, and were subsequently immune when challenged. Further evidence was acquired on the development of complement fixing antibodies in guinea pigs with an inapparent infection when 30 animals were inoculated with small doses of epidemic typhus rickettsiae. Seventeen of the animals responded in the usual manner. Thirteen individuals in the group developed no febrile reaction after inoculation but epidemic complement fixing antibodies were demonstrated in samples of serum obtained one month later from each of the thirteen. Finally, each of these animals proved to be immune when challenged with epidemic typhus.

Rocky Mountain Spotted Fever Thirteen guinea pigs were bled for serological studies and then inoculated intraperitoneally with 1 cc amounts of a pool of infectious blood obtained from guinea pigs acutely ill with a mild strain

of Rocky Mountain spotted fever. A febrile period of from 2 to 9 days followed an incubation period varying from 2 to 6 days. The guinea pigs were bled 14 days after the temperature returned to normal. Serum from each animal contained complement fixing antibodies that reacted with the Rocky Mountain spotted fever antigen and none that reacted with the epidemic or murine antigen. All pre-inoculation specimens were negative with the 3 antigens. Data from this experiment are summarized in Table III.

Discussion The identification of strains of epidemic and murine typhus or Rocky Mountain spotted fever based on animal reactions alone presents certain experimental difficulties. This has been illustrated when guinea pigs were inoculated at the same time with the same amount of a pool of infectious material. Irrespective as to whether the guinea pig develops evidence of disease as expressed by a febrile reaction or scrotal swelling, or an inapparent disease without these reactions, specific complement fixing antibodies develop in early convalescence. The use of the complement fixation reaction, likewise, permits the detection of those animals that represent missed infections or those that develop fever from non-specific causes. The use of the complement fixation method for strain identification is specific, rapid and inexpensive.

Summary 1 Guinea pigs inoculated intraperitoneally at the same time with the

same amount of a pooled infectious suspension of tunica vaginalis washings, Wilmington strain of murine typhus, developed three types of reaction following a variable period of incubation (a) fever of variable duration and scrotal swelling, followed by the appearance of specific murine complement fixing antibodies, (b) fever of variable duration and no scrotal swelling followed by the appearance of specific murine complement fixing antibodies, and (c) no febrile reaction or scrotal swelling but followed by the appearance of specific murine complement fixing antibodies (inapparent infection) All guinea pigs showing one of these three types of response were proven immune when challenged subsequently

2 Guinea pigs inoculated intraperitoneally at the same time with the same amount of pooled infectious brain suspension, Breml strain of epidemic typhus, developed 3 types of reaction following a variable period of incubation (a) febrile period of variable duration followed by the appearance of specific epidemic complement fixing antibodies, (b) no febrile reaction but followed by the appearance of specific epidemic complement fixing

antibodies (inapparent infection), and (c) no febrile reaction followed by no complement fixing antibodies This latter group are regarded as "misses" for these guinea pigs were not immune when challenged while those of group a and b were immune

3 Specific murine or epidemic complement fixing antibodies were demonstrated after the inapparent infections with these 2 types of typhus

4 Guinea pigs inoculated at the same time with the same amount of pooled infectious blood of Rocky Mountain spotted fever (mild strain) exhibit a febrile reaction of variable duration following a variable period of incubation In all instances specific complement fixing antibodies to Rocky Mountain spotted fever were demonstrated in convalescent specimens of serum and no complement fixing antibodies with a murine or epidemic antigen

Conclusion The specific complement fixation test, using purified rickettsial suspensions as antigen, is recommended as a routine test to differentiate infections in guinea pigs caused by rickettsiae of murine and epidemic typhus and Rocky Mountain spotted fever

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Studies on Chancroid IV The Ducrey Bacillus Growth Requirements and Inhibition by Antibiotic Agents *

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The classification of the Ducrey bacillus in the genus *Hemophilus* appears to be based largely on its morphology and on the fact that it grows best in a medium containing blood Actually the growth requirements and biology of this organism have received very little study The present article is a report of some observations made in the course of a clinical and laboratory investigation of chancroid The data presented support the view that

there are important differences between the Ducrey bacillus and other members of the genus *Hemophilus*

Materials and Methods The base medium used was proteose peptone broth (Difco) Blood was obtained from rabbits by cardiac puncture Defibrination was accomplished by shaking with glass beads Erythrocyte suspensions were prepared by washing repeatedly with large volumes of normal saline, until a negative Pandy test was obtained on the supernatant fluid

X factor was prepared as follows The clot

* Aided by a grant from the Venereal Disease Division of the United States Public Health Service

TABLE I

Growth of Ducrey Bacillus, *H influenzae*, and *H parainfluenzae*, in Media Containing Whole Blood and X and V Factors

Organism		Base medium, plus			
		X factor	V factor	X and V factors	Whole blood
Ducrey	No 1	0	0	0	++++
	No 2	0	0	0	++++
	No 3	0	0	0	++++
	No 4	0	0	0	++++
	No 5	0	0	0	++++
	No 6	0	0	0	++++
<i>H influenzae</i>	6208	0	0	++++	++++
"	"	0	0	++++	++++
"	"	0	0	++++	++++
<i>H parainfluenzae</i>	7901	0	++++	++++	++++

Base medium Proteose peptone broth (Difco)

Whole blood Defibrinated rabbit blood (concentration 10% in base medium)

H influenzae 6208, *H parainfluenzae* 7901 American Type Culture Collection*H influenzae* I, *H influenzae* II Type 1 strains recently isolated from patients with influenza meningitis

This table represents findings after more than three successive subcultures

Readings based on microscopic examination of stained smears of cultures, after 48 hours' incubation at 34°C

from 40 ml of rabbit blood was infused in 8 ml of normal saline at room temperature for one hour. This was then boiled for 5 minutes, and filtered through paper. The filtrate was autoclaved for 15 minutes at 15 pounds pressure. This material was added to the base medium in a concentration of 4% to provide an adequate supply of X factor.

V factor was prepared as follows. 20 g of baker's yeast were emulsified in 80 ml of distilled water, by rubbing in a glass mortar. The reaction was adjusted to pH 4.6 by addition of N/1 HCl. The mixture was boiled for 10 minutes and then centrifuged at 2000 rpm for 20 minutes. The clear supernatant material was used as a source of V factor, being added to the base medium in a concentration of 10%.

All cultures were incubated at 34°C. Liquid media were distributed in 1-ml quantities, and cultures were inoculated with 1 loopful of material.

Requirement of X and V Factors. *Hemophilus influenzae* has been shown to require for growth 2 agents which are present in erythrocytes. One of these, originally called the X factor, is heat-stable and is known to be hemin. The other, called V factor, is heat-labile, and is replaceable by coenzyme I or II.¹ *H para-*

influenzae requires only the V factor.

Six strains of Ducrey bacillus were tested for growth in the presence of X and V factors. Three strains of *H influenzae* and one of *H parainfluenzae* were included in the experiment. The results of the test are shown in Table I. It will be seen that all 10 organisms were able to grow in the medium in the presence of defibrinated blood. On the other hand, addition of X factor alone was not sufficient to support growth of any of the 10 organisms. *H parainfluenzae* was able to grow in a medium containing V factor alone. The 3 strains of *H influenzae* and *H parainfluenzae* all grew in a medium containing both X and V factors, but none of the Ducrey strains would grow in it. Thus, although the Ducrey bacillus resembles true members of the genus *Hemophilus* in that growth is supported by the presence of blood in the medium, it differs by inability to grow in a medium containing only X and V factors. One must conclude from this either that the X and V factors are not the substances essential for growth of the Ducrey bacillus, or that some other factor which is present in whole blood in addition to X and V factors is needed for growth.

Growth in Media Containing Cells Alone, or Serum Alone. The constituents of whole blood necessary for the growth of *H influenzae* and *H parainfluenzae* are present in the ery-

¹ Lwoff, A., and Lwoff, M., *Proc Roy Soc, London, Series B*, 1937, **122**, 352, 360.

TABLE II

Growth of Ducrey Bacillus, *H influenzae* and *H parainfluenzae* in Media Containing Blood Serum or Washed Erythrocytes

Organism	Base medium, plus	
	Blood serum	Washed erythrocytes
Ducrey No 1	++++	++++
No 2	++++	++++
No 3	++++	++++
No 4	++++	++++
No 5	++++	++++
No 6	++++	++++
<i>H influenzae</i> 6208	0	++++
" " I	0	++++
" " II	0	++++
<i>H parainfluenzae</i> 7901	0	++++

This table represents findings after more than 3 successive subcultures

Readings based on microscopic examination of stained smears of cultures, after 48 hours' incubation at 34°C

throcytes, not in the blood serum. It is known, however, that the Ducrey bacillus will grow in a medium enriched only with blood serum. A demonstration of this is the experiment shown in Table II. The concentration of serum required for growth of Ducrey bacilli appears to be relatively high. Optimum growth was obtained in media containing from 25 to 50% serum although some of the strains tested survived when the concentration was reduced to as low as 10%. The fact that Ducrey bacilli will grow in serum broth, a medium which will not support growth of either *H influenzae* or *H parainfluenzae*, together with the evidence in the preceding section, suggests that neither X nor V factor is essential for growth of the Ducrey organism.

The Ducrey bacillus can also be cultivated in a base medium enriched only with erythrocytes. The volume of erythrocytes necessary is relatively large, 10% or greater.

It would appear that enrichment with whole blood provides a better medium for growth of the Ducrey bacillus than is obtained with either cells or serum alone since growth can be maintained indefinitely in a base medium containing only 3% of defibrinated blood. It is probable, therefore, that whole blood contains more than one growth substance utilized by Ducrey organisms, and that these substances are present in both erythrocytes

and serum. Further evidence bearing on this will be given in the next section.

Heat Stability of Growth Factors in Erythrocytes and Serum. The substances in rabbit blood serum essential for growth of Ducrey bacilli are not destroyed by heating at 100°C for 30 minutes. Twenty per cent serum broth heated in this manner will support the growth of Ducrey bacilli through repeated transfers without apparent change. Growth does not occur, however, in serum broth which has been autoclaved at 15 pounds pressure for 20 minutes.

The growth-supporting properties of erythrocytes are more easily destroyed by heat than are those of serum. Ten per cent erythrocyte-broth would not support growth of any of 6 strains of Ducrey bacillus after heating at 65°C for 30 minutes. Four of the 6 strains tested would not grow in this medium after it had been heated at 60° for 30 minutes.

The difference in heat stability of the growth factors in erythrocytes and serum provides further evidence that the agents present in the two constituents of blood are not the same.

Importance of Moisture for Growth of Ducrey Organisms on Solid Media. A striking peculiarity of members of the Ducrey group is their dependence on moisture for growth on the surface of a solid medium. We have discussed this elsewhere, in an article dealing with the cultural diagnosis of chancroid.² Usually no visible growth occurs on the surface of an inoculated blood agar plate placed in an ordinary incubator. On the other hand, a plate which has received a similar inoculum and which is incubated at the same temperature inside a closed jar containing a moist sponge will show a good growth of Ducrey colonies at the end of 24 hours. *H influenzae* and *H parainfluenzae* show no such rigid dependence on moisture in the air.

Satellite Phenomenon. Most members of the genus *Hemophilus* exhibit more luxuriant growth on the surface of a solid medium in the vicinity of colonies of certain other bacteria, particularly staphylococci. This enhanced growth is called the "satellite phe-

² Beeson, P. B., and Heymann, A., *Am J Syph, Gon and Ven Dis.*, in press.

TABLE III

Growth of Ducrey Bacillus, *H. influenzae*, and *H. parainfluenzae* in Media Containing Varying Concentrations of Tyrothricin

Organism	Dilution of tyrothricin						
	4000	8000	16000	32000	64000	128000	0
Ducrey No 1	0	0	0	+	+++	+++	++++
No 2	0	0	0	0	++	++++	++++
No 3	0	0	0	0	++	++++	++++
No 4	0	0	0	0	0	++	++++
No 5	0	0	0	++	+++	+++	++++
No 6	0	0	0	++	++	++++	++++
<i>H. influenzae</i> 6208	0	++	++++	++++	++++	++++	++++
" " I	0	++	++++	++++	++++	++++	++++
" " II	0	+++	++++	++++	++++	++++	++++
<i>H. parainfluenzae</i> 7901	+	++	++++	++++	++++	++++	++++

Medium 10% defibrinated blood in base medium

This table represents findings after more than three successive subcultures

Readings based on microscopic examination of stained smears of cultures, after 48 hours incubation at 34°C

nomenon" It is thought to be due to liberation of growth substances by the staphylococci. No such change is noted in Ducrey colonies, whose gross appearance is not altered by proximity to staphylococcus colonies on the surface of a solid medium.

Dialysis of Growth Factors Repeated attempts were made to demonstrate the growth factors for Ducrey organisms in dialysates of either defibrinated rabbit blood or fresh rabbit serum. Blood and serum were dialyzed against distilled water, physiologic salt solution, and proteose peptone broth. Sterilization of dialysates was accomplished by Seitz filtration, a procedure which does not affect the growth-stimulating properties of serum broth. None of the dialysates obtained would support growth, either alone or when added to the base medium.

Bacteriostasis by Antibiotic Substances The 3 strains of *H. influenzae* and 1 strain of *H. parainfluenzae* were compared with 6 strains of Ducrey bacillus in respect to inhibition of growth by penicillin, tyrothricin, streptomycin,[†] and streptothricin. Other workers have already reported that penicillin exerts a pronounced bacteriostatic effect on Ducrey organisms.³ We have repeated that work and our findings agree entirely. In addition we

find a similar difference in respect to inhibition by tyrothricin. The results of a test are shown in Table III, where it will be observed that the typical *Hemophilus* organisms were able to grow in much greater concentrations of tyrothricin than was the case with strains of Ducrey bacillus. This is further evidence of a basic difference between Ducrey bacilli and true *Hemophilus* organisms. In respect to streptothricin and streptomycin, no significant difference in susceptibility was found.

Summary In a comparative study of several strains of Ducrey bacillus with *H. influenzae* and *H. parainfluenzae* a number of significant biologic differences have been noted. The Ducrey bacillus will not grow in a medium which supplies both the X and V factors. Unlike these other members of the genus *Hemophilus*, the Ducrey bacillus will grow in a medium enriched only with blood serum. It will also grow in a medium enriched only with erythrocytes. The agents in serum and in erythrocytes which support growth of the Ducrey bacillus differ in heat stability, that in serum withstands 100°C but not autoclaving, while that in erythrocytes is inactivated by heating to 65°C. The addition of both erythrocytes and serum to the base medium provides a more favorable medium for the growth of the Ducrey bacillus than either one alone. Ducrey colonies will not develop on the surface of a solid medium unless the humidity of the atmosphere is very

[†] Courtesy of Dr S. A. Waxman

³ Mortara, M., Feiner, R. R., and Levenhron, E., *Proc. Soc. Exp. Biol. and Med.*, 1944, **56**, 163

high The "satellite phenomenon" is not observed in colonies of Ducrey bacillus Attempts to separate Ducrey growth factors from whole blood or serum by dialysis have not been successful The Ducrey bacillus

differs significantly from *H. influenzae* and *H. paramfluenzae* in susceptibility to inhibition by penicillin and by tyrothricin

Miss Elizabeth Roberts gave technical assistance in this work

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Muscles Consisting of a Single Motor Unit After Poliomyelitis

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During an investigation concerning the possible increase of muscle force in paretic muscles by means of increased motor nerve branching¹ the action potentials of more than 1000 different paretic muscles were obtained Among these the records of 36 muscles in 26 different patients were remarkable for their simplicity and they resembled closely records produced by single motor units in fully^{2,3,4} or partially innervated muscles,⁵ though no measures were taken to insure the recording of units The behavior of these muscles was analyzed by repeated tests

Methods In routine electrograms, the patient contracted the muscle as strongly as possible for 2 or 3 seconds and then released the tension quickly Electrodes, consisting of solder pellets of 5 mm diameter were attached to the skin with tape, electrode paste being used to insure a good contact The action potentials were amplified and recorded by a Matthews oscillograph Nearly all of the muscles to be described were found in patients who had undergone the treatments intended to enhance the nerve branching¹ In all cases the paresis was at least of one year standing

Results The short lasting, strong con-

tractions of all muscles under consideration were accompanied by a single row of action potential spikes, (Fig. 1) which were in general, rather evenly spaced and often grew considerably in height, especially the first 10 spikes In by far the majority of the cases the voluntary control of the muscle was good, in only a few were there signs of poor control as evidenced by an irregular and low frequency of the spikes and difficulty in maintaining the contraction even for a short time

The maximum frequency of the spikes reached in this way has been measured for all these muscles and was found to vary between 20 and 60 per second, the mean being 40 In accordance with the data of Gibson and Mills³ it was observed that 2 or even 3 spikes could follow each other closely, (0.01 second), after which there was usually a somewhat longer delay before the appearance of the next spike Therefore the maximum frequency was computed by using the time interval of at least 10 consecutive spikes Since most of the 15 different muscles used in the investigation are represented only a few times and these are only larger muscles in which the influence of the contraction of neighboring muscles on the electrogram is negligible the material is not very suitable to show whether or not different muscles have different inherent maximum frequencies It may be significant, however, that the deltoid which is represented 5 times showed a maximum of 60 in 3 and of 40 in 2 of these cases, and has thus a mean frequency greater than that of the mean maximum given above

¹ Billig, H. E., van Haterfeld, A., and Wiersma, C. A. G., *J. Neuropath. and Exp. Neurol.* 1946, in press

² Smith, O., *Am. J. Physiol.* 1934, **108**, 629

Gilson, A. S. and Mills, W. B., *Am. J. Physiol.* 1941, **133**, 678

⁴ Denslow, J. S. and Hisscott, C. C., *Am. J. Physiol.* 1943, **139**, 652

⁵ Fimdes, D. B., *Am. J. Physiol.* 1935, **114**, 90

TABLE III
Growth of Ducrey Bacillus, *H. influenzae*, and *H. parainfluenzae* in Media Containing Varying Concentrations of Tyrothricin

Organism	Dilution of tyrothricin						
	4000	8000	16000	32000	64000	128000	0
Ducrey No 1	0	0	0	+	+++	+++	++++
No 2	0	0	0	0	++	++++	++++
No 3	0	0	0	0	++	+++	++++
No 4	0	0	0	0	0	++	++++
No 5	0	0	0	++	+++	+++	++++
No 6	0	0	0	++	++	++++	++++
<i>H. influenzae</i> 6208	0	++	++++	++++	++++	++++	++++
" " I	0	++	++++	++++	++++	++++	++++
" " II	0	+++	++++	++++	++++	++++	++++
<i>H. parainfluenzae</i> 7901	+	++	++++	++++	++++	++++	++++

Medium 10% defibrinated blood in base medium

This table represents findings after more than three successive subcultures

Readings based on microscopic examination of stained smears of cultures, after 48 hours incubation at 34°C

nomenon" It is thought to be due to liberation of growth substances by the staphylococci. No such change is noted in Ducrey colonies, whose gross appearance is not altered by proximity to staphylococcus colonies on the surface of a solid medium.

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Bacteriostasis by Antibiotic Substances The 3 strains of *H. influenzae* and 1 strain of *H. parainfluenzae* were compared with 6 strains of Ducrey bacillus in respect to inhibition of growth by penicillin, tyrothricin, streptomycin,[†] and streptothricin. Other workers have already reported that penicillin exerts a pronounced bacteriostatic effect on Ducrey organisms.³ We have repeated that work and our findings agree entirely. In addition we

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Summary In a comparative study of several strains of Ducrey bacillus with *H. influenzae* and *H. parainfluenzae* a number of significant biologic differences have been noted. The Ducrey bacillus will not grow in a medium which supplies both the X and V factors. Unlike these other members of the genus *Hemophilus*, the Ducrey bacillus will grow in a medium enriched only with blood serum. It will also grow in a medium enriched only with erythrocytes. The agents in serum and in erythrocytes which support growth of the Ducrey bacillus differ in heat stability, that in serum withstands 100°C but not autoclaving, while that in erythrocytes is inactivated by heating to 65°C. The addition of both erythrocytes and serum to the base medium provides a more favorable medium for the growth of the Ducrey bacillus than either one alone. Ducrey colonies will not develop on the surface of a solid medium unless the humidity of the atmosphere is very

[†] Courtesy of Dr S. A. Waxman

³ Mortara, M., Feiner, R. R., and Leveukion, E., *Proc. Soc. Exp. Biol. and Med.*, 1944, 56, 163

high The "satellite phenomenon" is not observed in colonies of Ducrey bacillus Attempts to separate Ducrey growth factors from whole blood or serum by dialysis have not been successful The Ducrey bacillus

differs significantly from *H influenza* and *H paramyxa* in susceptibility to inhibition by penicillin and by tyrothricin

Miss Elizabeth Roberts gave technical assistance in this work

15233

Muscles Consisting of a Single Motor Unit After Poliomyelitis

C A G WIERSMA

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During an investigation concerning the possible increase of muscle force in paretic muscles by means of increased motor nerve branching¹ the action potentials of more than 1000 different paretic muscles were obtained Among these the records of 36 muscles in 26 different patients were remarkable for their simplicity and they resembled closely records produced by single motor units in fully^{2,3,4} or partially innervated muscles,⁵ though no measures were taken to insure the recording of units The behavior of these muscles was analyzed by repeated tests

Methods In routine electrograms, the patient contracted the muscle as strongly as possible for 2 or 3 seconds and then released the tension quickly Electrodes, consisting of solder pellets of 5 mm diameter were attached to the skin with tape, electrode paste being used to insure a good contact The action potentials were amplified and recorded by a Matthews oscillograph Nearly all of the muscles to be described were found in patients who had undergone the treatments intended to enhance the nerve branching¹ In all cases the paresis was at least of one year standing

Results The short lasting, strong con-

tractions of all muscles under consideration were accompanied by a single row of action potential spikes, (Fig 1) which were in general, rather evenly spaced and often grew considerably in height, especially the first 10 spikes In by far the majority of the cases the voluntary control of the muscle was good, in only a few were there signs of poor control as evidenced by an irregular and low frequency of the spikes and difficulty in maintaining the contraction even for a short time

The maximum frequency of the spikes reached in this way has been measured for all these muscles and was found to vary between 20 and 60 per second, the mean being 40 In accordance with the data of Gibson and Mills² it was observed that 2 or even 3 spikes could follow each other closely, (0.01 second) after which there was usually a somewhat longer delay before the appearance of the next spike Therefore the maximum frequency was computed by using the time interval of at least 10 consecutive spikes Since most of the 15 different muscles used in the investigation are represented only a few times and these are only larger muscles in which the influence of the contraction of neighboring muscles on the electrogram is negligible the material is not very suitable to show whether or not different muscles have different inherent maximum frequencies⁷ It may be significant, however that the deltoid which is represented 5 times showed a maximum of 60 in 3 and of 40 in 2 of these cases and has thus a mean frequency greater than that of the mean maximum given above

¹ Billig, H F van Harreveld, A and Wiersma, C A G, *J Neuropath and Exp Neurol* 1946, in press

² Smith, O *Am J Physiol*, 1934, 108 629

Gilson, A S, and Mills, W B, *Am J Physiol*, 1941 133, 678

⁴ Dunslow, J S and Hasset, C C *Am J Physiol* 1943 139, 652

Indslev, D B, *Am J Physiol* 1935 114 90

TABLE III
Growth of Ducrey Bacillus, *H. influenzae*, and *H. parainfluenzae* in Media Containing Varying Concentrations of Tyrothricin

Organism	Dilution of tyrothricin						
	4000	8000	16000	32000	64000	128000	0
Ducrey No 1	0	0	0	+	+++	+++	++++
No 2	0	0	0	0	++	++++	++++
No 3	0	0	0	0	++	+++	++++
No 4	0	0	0	0	0	++	++++
No 5	0	0	0	++	+++	+++	++++
No 6	0	0	0	++	++	++++	++++
<i>H. influenzae</i> 6208	0	++	++++	++++	++++	++++	++++
" " I	0	++	++++	++++	++++	++++	++++
" " II	0	+++	++++	++++	++++	++++	++++
<i>H. parainfluenzae</i> 7901	+	++	++++	++++	++++	++++	++++

Medium 10% defibrinated blood in base medium

This table represents findings after more than three successive subcultures

Readings based on microscopic examination of stained smears of cultures, after 48 hours incubation at 34°C

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[†] Courtesy of Dr. S. A. Waxman.

³ Mortara, M., Feiner, R. R., and Levenkron, E., *Proc. Soc. Exp. Biol. and Med.*, 1944, **56**, 163.

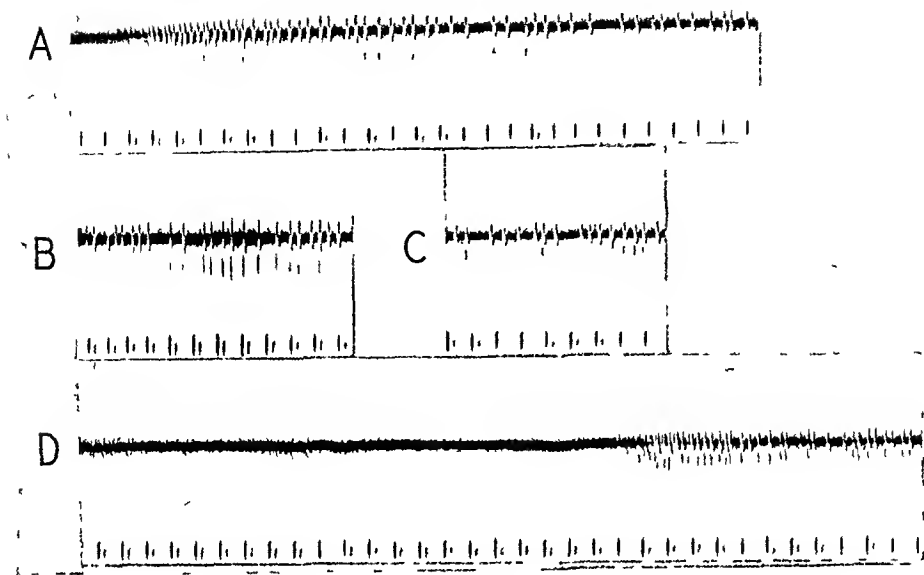


FIG 2

Deltoid, tired. Long lasting contraction. A Start. Notice pronounced fibrillation and triphasic spikes. B After 15 seconds, maximum fibrillation is reached. C After 30 seconds, the height of the spikes is diminished. D After 90 seconds. The contraction ends with muscle action potentials just visible. After 1 second rest they reappear and fibrillate, but diminish quickly. Time 1/10 second.

which had been rested for a long time reached maximum peak height after one minute, whereas the same muscle normally reached its maximum after 30 seconds and already began to diminish in height at one minute. If, therefore, a patient has used the muscle to a great extent, *e.g.* in walking, the rest provided during the investigation time may not be long enough to overcome the after-effects of this use.

In another series of experiments repeated contractions of very short duration were studied. In such contractions each ensuing contraction starts with a higher action potential than the previous one (Fig 3) which shows that purely mechanical factors are not responsible for the increase in height. In this type of contraction the frequencies reached tend to be higher than during the "standard" contractions and may become high enough to seriously interfere with the gradual growth of the action potentials (Fig 3). At least in these muscles it seems possible to obtain

frequencies in the nerve fibers which are too high for the muscle fibers to follow normally.

In a number of selected cases mechanograms were obtained from these muscles by the application of a heart apex beat recorder over the contracting belly. In some cases but not in all, single twitches caused by a single impulse were obtainable,³ in these it was found that higher contractions and action potentials occurred after previous facilitation by a tetanic contraction. Summations of 2 and more impulses could be nicely demonstrated with this method (Fig 4).

In some of the cases needle electrodes were put into the substance of the muscle. The resulting action potentials differed only in voltage from those obtained from the intact skin.

Discussion As in all cases of human muscles it is not certain that the data presented are caused by the action of single motor units. Especially the growth of the

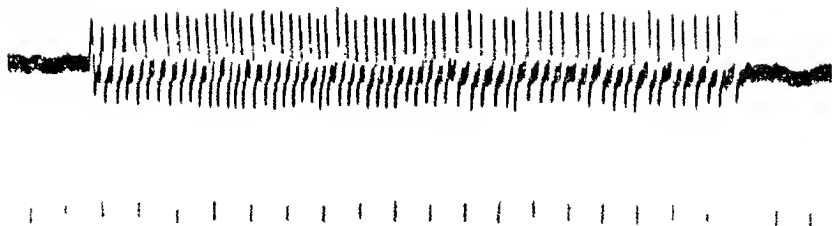


FIG 1

Test contraction of rectus femoris, unfatigued patient Facilitation 25%,
 diphasic frequency 40 per second As in all following pictures skin leads were used
 Time 1/10 sec

The growth of the spikes of the action potentials during the contraction was present in all cases and often very pronounced. In records in which the action potentials were practically monophasic this increase was usually larger than with di- or polyphasic spikes. In the latter there was an increase of more than 50% in 14 of the 27 records, and in only 1 record was the increase as small as 20%. In the monophasic group 13 out of 26 records showed an increase of more than 100% and all but one of more than 50%. One muscle was outstanding in the amount of facilitation obtained. The first top was always very small and the later ones were at least 10 times as large (Fig 2).

The amount of facilitation was found to be dependent on several factors, in any one muscle. Relatively little difference was encountered when on different days similar contractions were performed with similar electrode placements, unless the state of fatigue of the muscle was different. At any given time with the electrodes fixed, the most important factor was the frequency of the impulses. Up to a certain limit the growth is faster and larger the more rapidly the spikes follow each other. However, above a certain frequency, which seems to differ for different muscles, the potentials become smaller. This does not interfere with the facilitation process, any spike which happens to be separated by a sufficient time interval from a foregoing one will be large. In a

fatigued muscle the growth is much smaller and may, soon after the start of the contraction, stop and be replaced by a decrease in height, even when the frequency is low. In a few instances the muscle was in tone both before and after the test contraction. In these cases little or no facilitation took place, which is explained by the supposition that during the tonic period facilitation had developed to such an extent, that the increase in frequency has no longer a pronounced effect.

In a limited number of experiments the contractions were maintained for long times. It was found that in most cases the spikes after their initial facilitation start to decline confirming Smith² and may become very small indeed (Fig 2). In others the frequency becomes very irregular which in turn gives irregular action potential heights. In one case 5 minutes contraction did not result in any noticeable decrease. Though the rate of the spikes was rather low, (± 25), other muscles with even lower rates showed profound fatigue after much shorter times.

Once a muscle has been fatigued, the result remains noticeable for a long time. During the first minutes after relaxation the action potentials remain small, after longer rest periods the growth is at first normal but diminishes soon and the maximum height is never reached. This may well play a part in the difference of facilitation obtained in different muscles. In one instance a muscle

every impulse. Such a mechanism has been claimed to be normal in avian muscles.⁶ Some experiments were performed with this possibility in mind. The patients were given prostigmine, intramuscular in the acute and orally in other experiments. The dosage was sufficient to cause distinct signs of the action of the drug, of which especially the increase in the size of the action potentials in the acute cases should be mentioned. However, no noticeable effect on the degree of

facilitation was observable. It must therefore remain at this time undecided by which mechanism the facilitation is brought about.

Summary. It was found that in some patients with chronic poliomyelitis very weak muscles can be found which seem to consist of one remaining motor unit. A study of their properties has been made by recording the action potentials through skin leads.

The author is indebted to Dr. A. van Hureveld and Dr. H. E. Billig for their help and for their interest in this research.

⁶ Brown, G. L., and Harvey, A. M., *J. Physiol.*, 1938, **93**, 285.

15234 P

Development of Brucella Agglutinins in Humans Following Vaccination for Cholera *

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During the course of a survey for brucella agglutinins made on sera discarded from the Wassermann laboratory (C. W. E., N. B. McC. and G. A. B.), a blood bank donor was found to have a brucella agglutinin titer of 1:200. This individual was a healthy male who, 3 months previously, had been experimentally vaccinated by one of us (W. B.) with U. S. Army cholera vaccine. The possibility that this finding might be more than a coincidental occurrence led us to examine the stored sera from the cholera studies for brucella agglutinins.

Methods and Material. Thirty-four individuals were given either regular U. S. Army cholera vaccination or varying amounts of an experimental cholera vaccine consisting of extracted cell residue. Whereas the Army vaccine contained both H and O antigens,

the experimental vaccine contained primarily the O antigen. The vaccines were given in 2 doses of 0.5 and 1.0 cc with an interval of one week between inoculations. Sera were collected before immunization and at 1, 2, 5 and 9 weeks after completion of the vaccination. Brucella agglutinin titers were determined by the rapid slide method of Huddleson, using the standard dilutions from 1:25 to 1:500. The subjects were divided into 4 groups.

Group I—7 subjects, routine U. S. Army cholera vaccination, 8,000 million organisms per cc.

Group II—8 subjects, experimental vaccine, 8,000 million organisms per cc.

Group III—10 subjects, experimental vaccine, 24,000 million organisms per cc.

Group IV—9 subjects, experimental vaccine, 40,000 million organisms per cc.

Five post-immunization sera from 3 individuals were further studied by reciprocal agglutinin absorption tests, using as antigens a live virulent strain of *Br. abortus* and a live 24-hour culture of *Vibrio comma*. After absorption with brucella, the sera were tested

* Aided in part by a grant from Swift and Company. A part of the work in this paper was done under a contract, recommended by the Committee on Medical Research between the Office of Scientific Research and Development and the University of Chicago.

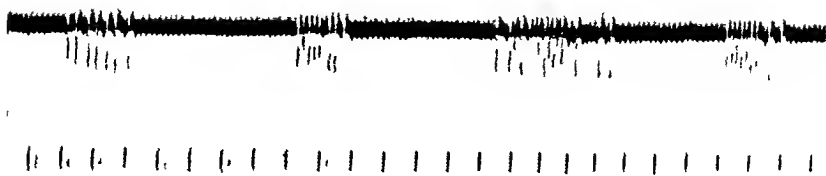


FIG 3

Lower peroneal, treated. Series of short listing contractions. In the first of the series the facilitation is regular. In the following three the frequency is higher and the spikes are irregular. Each of the later contractions starts with a higher spike than the previous one. Time 1/10 sec.

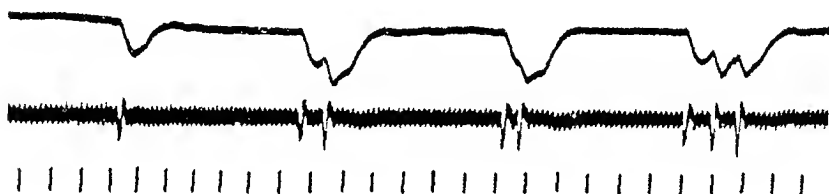


FIG 4

Gluteus maximus, treated. A series of voluntary twitches, the first of which is single. Upper line, myoelectric, middle electrogram, lower time 1/10 sec.

action potentials which from the evidence presented cannot be ascribed to artifacts, might well be considered as against the presence of single motor units. The alternative would be that a number of nerve fibers would always fire at the same time, but would have different thresholds. Such a mechanism might possibly be brought about by the connection of a number of motoneurons with a single pyramidal fiber. If this were the case the number of motoneurons so connected should be large in order to explain the smooth increase of the growth of the potentials. No evidence was ever obtained which showed that these hypothetical central units could split themselves into component parts, and they would therefore form a serious handicap to the amount of central control of the contractions. Furthermore in all cases the contracting part of the muscle was a single band, whereas if different axons were involved one would expect that several contractile bands would occur in at least some of them.

If then these bands are considered as single units it remains to be discussed what the growth in the action potentials represents. There are two likely explanations. The first is that facilitation represents the increased spreading of the action potential over the muscle fibers. In freshly reinnervated muscles such a process might well take place, the first impulse of a series would be limited to the neighborhood of the end plate and successive impulses would spread more and more to the ends of the muscle fibers. However, as recovery went on, it would be expected that facilitation become less and less pronounced. This was not observed, in patients which have been studied for one year or more no difference in the amount of facilitation was found between the first and the last test. The other, and perhaps more likely possibility is that only part of the muscle fibers respond on a single nerve impulse and that considerable facilitation is necessary before all fibers contract with

for brucellosis. Although it is well known that proven cases of the disease may at times present little or no agglutinin response, the diagnosis of brucellosis is frequently made on the basis of low-grade titers without adequate supporting data. Many clinicians consider brucella agglutinin titers in dilutions of 1:500 as almost certainly diagnostic of active infection. The development of titers as high as this following vaccination for cholera may well be expected to introduce further confusion in the clinical interpretation of this test. Cholera vaccination has been infrequently used in this country prior to the war, but large numbers of veterans are returning from the Pacific and Asiatic

theaters who have been subjected to this immunization.

Studies are underway to determine the duration of the persistence of brucella antibodies following vaccination for cholera, and the possible reappearance of antibodies in the circulating blood as an anamnestic phenomenon. The possible effect of other types of immunizations on brucella antibody stimulation must also be considered.

Our preliminary data presented here indicate that there is an H antigen in *Vibrio comma* which is present also in brucella. Studies are in progress to elucidate further the antigenic interrelationships of these organisms.

15235 P

Excitability of Muscle to Direct and Indirect Stimulation During Prolonged Direct Stimulation*

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Although electrical stimulation is used widely in diagnosis and in physical therapy and is the only practical procedure to test muscle function in animals, the effects of prolonged direct stimulation have not been studied extensively.

Alternating 60-cycle current was used in 3 independent but *in phase* circuits for stimulation through 3 pairs of electrodes, the first pair being inserted into the knee and tendon of the gastrocnemius muscle *in situ* of anesthetized rats (nembutal) and cats (dial), (D_1 electrodes), the second pair (D_2) inserted into the muscle itself at its upper and lower ends, the third pair (I) being employed for stimulation of the peripheral end of the cut sciatic nerve. The strength of the stimulus was adjusted with a rheostat and measured by a volt meter, in addition, the resistance in the muscle was measured by means of an oscilloscope in a shunt parallel to the muscle. Stimulation was continued either with D_1 or with D_2

until the muscle length reached the pre-stimulus resting value. During and after the continued stimulation, brief tetanic stimuli were applied at regular intervals through the 2 other pairs of electrodes. In all cases maximum stimuli were applied through a 10,000-ohm resistance placed in series with muscle and nerve. The muscle was loaded with 200 g and contraction was isotonic. Before continued stimulation the effect of summation was tested for all combinations of D_1 , D_2 , and I. There was no summation between D_1 and I, or D_2 and I, and a tendency to slight summation of $D_1 + D_2$.

Steel, platinum, silver, and nonpolarizable (Cu-CuSO_4 , 0.1M - K_2SO_4 , 0.1M - NaCl , 0.1M NaCl , 4% - Tissue) electrodes were employed in various experiments. There was no essential difference in the results to be reported. Forty-seven experiments were performed in rats and 3 experiments in cats.

With continued stimulation through D_1 , there is no response to D_2 or I at the very beginning but after a few minutes a marked

* Aided by a grant from the National Foundation for Infantic Paralysis, Inc.

TABLE I
Brucella Agglutinins in Sera of Subjects Immunized with Regular U S Army Cholera Vaccine

Subject No	Pre immunization sera	Time after last inoculation			
		1	2	5	9 weeks
7	—	1 500P	1 200	1 200P	1 200P
10	—	1 500P	1 500	1 200	1 200
16	1 25	1 25	1 50	1 50	*
21	—	—	—	—	—
30	—	—	1 100P	—	—
32	—	1 200P	1 100P	1 50	1 25
38	—	1 100P	1 25	—	—

— Negative

P Partial agglutination (usually complete agglutination in the next lower titer)

* Serum not available

with cholera antigens¹ containing HO and containing only O antigen

Results The brucella agglutinin titers of the 7 individuals in Group I who received the U S Army type of cholera vaccination are presented in Table I. One of the subjects had a pre-immunization titer of 1 25, all the others being negative. Following immunization, all except one developed brucella agglutinins in titers ranging from 1 50 to 1 500. In 3 subjects, brucella agglutinins were still present at the time of the last collection of sera (9 weeks).

In Groups II, III and IV receiving varying amounts of the experimental cholera vaccine, none of the 27 individuals showed any pre-immunization brucella titers, and in only 2 subjects, low-grade post-immunization brucella agglutinin titers developed. These were both in Group IV receiving the largest amount of the vaccine, and the titers of 1 25 and 1 50 P were very transient, disappearing by the 9th week.

In the reciprocal agglutinin absorption tests, absorption with living 24-hour culture of *Vibrio comma* removed all brucella agglutinins. Sera absorbed with a live, virulent strain of *Brucella abortus* subsequently agglutinated formalinized *Vibrio comma* (HO) in markedly lowered titers[†] (Serum E7, before absorption 1 1000, after absorption 1 100, Serum B32, before 1 5000, after 1 200, Serum C10, before 1 5000, after 1 200, Serum C32, before 1 5000, after 1 500, Serum D7, before 1 2000, after

1 2000[†]). Agglutination tests with a boiled suspension of *Vibrio comma* (O antigen) were not significantly different from the pre-absorption tests.

All 34 of the vaccinated subjects developed cholera agglutinins in high titers which persisted during the 9-week post-immunization observation period. The magnitude of the cholera agglutinin response was approximately the same following the two types of vaccine. In the 3 groups receiving the varying amounts of the experimental vaccine, augmented cholera agglutinin titers did not accompany increasing amounts of vaccine. In fact, Group IV receiving the largest amount of vaccine had a somewhat poorer average cholera agglutinin response than Groups II and III receiving smaller amounts. These data will be reported in detail elsewhere¹.

Discussion Wong and Chow² reported that in 8 rabbits injected intravenously or subcutaneously with heat-killed suspensions of *Vibrio comma*, brucella agglutinins developed in unspecified titers. Of 6 humans inoculated subcutaneously or intravenously with a typhoid-cholera vaccine, 4 developed agglutinins against *Brucella abortus* in titers of 1 40 or less. Presumably, the vaccines used had lost at least some of the H antigen during heating.

The agglutination test for brucella is the most commonly used diagnostic procedure

[†] In one serum, D7, the post absorption titer was not lowered. Insufficient serum remained for further tests.

² Wong, D H, and Chow, C H, *Chinese Med J*, 1937, 32, 591.

B₁₁ activity but low in folic acid can promote excellent growth and feathering in the chick. Piffner *et al*⁶ isolated a crystalline compound from liver which they called vitamin B_c and found that it has both growth-promoting and hemopoietic properties for the chick. Since vitamin B_c also has growth-promoting properties for *Lactobacillus casei*, these workers suggested that vitamin B_c, Hutchings' norite eluate factor, and Williams' folic acid⁷ are probably identical. Binkley and coworkers⁸ isolated from yeast a B_c conjugate which has vitamin B_c activity for the chick but no activity for the bacterium. However, upon enzymatic digestion the B_c conjugate becomes active as a source of B_c for the *Lactobacillus casei*. Day and coworkers⁹ reported that highly purified *Lactobacillus casei* factor was effective in treating vitamin M-deficient monkeys. In this paper we wish to report the relative effectiveness of vitamin B₁₀ and B₁₁ concentrates, vitamin B_c, vitamin B_c con-

jugate and the synthetic *Lactobacillus casei* factor when used in place of the norite eluate concentrate in our purified rations for the monkey.

Experimental The method of handling the monkeys has been described previously.¹⁰ The M-2 basal diet consisting of sucrose 73, Labco casein 18, salts IV 4, corn oil 2, cod liver oil 3 was fed *ad libitum* and supplemented with adequate amounts of ascorbic acid, thiamine, riboflavin, nicotinic acid, pyridoxine, choline, pantothenic acid, inositol, *p*-aminobenzoic acid, and biotin.

A liver fraction high in B₁₀ and B₁₁ activity but low in "folic acid" activity was prepared according to the directions of Briggs *et al*.¹¹ In brief this method consists of dialysing a concentrated superfiltrol eluate² in a cellophane bag against cold water for 96 hours. The residue is saved and placed in a Visking cellulose casing and dialysed against hot distilled water. The dialysate is saved and concentrated so that 1 cc of the final solution is equivalent to 10 g of the original solubilized liver extract (fraction L).

The dialysate was assayed for B₁₀ and B₁₁ activity using chicks and for "folic acid" using the microbiological method. Using as a standard (100%) the growth and feathering of chicks fed a ration containing 2% solubilized liver (fraction L), one cc of the dialysate showed 95% B₁₀ and B₁₁ activity. Assay with *Streptococcus lactis* showed that 1 cc of the dialysate contained less than 10 γ folic acid using crystalline vitamin B_c as the standard.

Studies with B₁₀ and B₁₁ Concentrates Three monkeys, No. 217, 218, and 219, were placed on the basal ration and given daily 1 cc of the concentrated dialysate. The monkeys grew at a slow rate for 8 weeks after which they began to lose weight. The supplement was then increased to 2 cc per day equivalent to 20 g of the original solubilized liver. This too proved to be inadequate and within two weeks the monkeys became cachectic and developed anorexia. Monkeys 217 and 219 were

Hutchings of Lederle Laboratories Inc. Pearl River, N Y, for the synthetic *Lactobacillus casei* factor, to Mrs Edith Jones for assistance in the differential leucocyte counts, to Mr T D Luckey for the B₁₀ and B₁₁ assays, and to Mrs Lillian Alberty for the folic acid assays.

¹ Wasmann, H A, and Elvehjem, C A, *J Nutrition* 1943, **26**, 361.

² Hutchings, B L, Bohonos, N, and Peterson, W H, *J Biol Chem* 1941, **141**, 521.

³ Hutchings, B L, Bohonos, N, Hegsted, D M, Elvehjem, C A, and Peterson, W H, *J Biol Chem*, 1941, **140**, 681.

⁴ Briggs, G M, Jr, Luckey, T D, Elvehjem, C A, and Hart, L B, *J Biol Chem* 1943, **148**, 163.

⁵ Briggs, G M, Jr, Luckey, T D, Elvehjem, C A, and Hart, L B, *J Biol Chem* 1945, **158**, 303.

⁶ Piffner, J J, Binkley, S B, Bloom, E S, Brown, R A, Bird, O D, Emmett, A D, Hogran, A G, and O'Dell, B L, *Science* 1943, **97**, 404.

⁷ Mitchell, H K, Snell, E E, and Williams, R I, *J Am Chem Soc*, 1941, **63**, 2284.

⁸ Binkley, S B, Bird, O D, Bloom, E S, Brown, R A, Calkins, D G, Campbell, C J, Emmett, A D, and Piffner, J J, *Science* 1944, **100**, 36.

⁹ Day, P L, Mims, A, Totter, I R, Stokstad, I L R, Hutchings, B L, and Sleime, N H, *J Biol Chem*, 1945, **157**, 423.

¹⁰ Wasmann, H A, Rasmussen, A F, Jr, Elvehjem, C A, and Clark, P F, *J Nutrition*, 1945, **26**, 205.

response to D_2 appears, while response to I is absent. The absence of response to I cannot be due to the absence of muscle fibers available for stimulation, since response to D_2 is present. After 10 to 20 minutes of stimulation with D_1 , response to I begins to reappear and to increase gradually in size, but usually remains somewhat smaller than the D_2 response. After "fatigue" (i.e., approach to the original resting length) the responses to both I and D_2 are very marked and may approach the height of initial contraction. If at this stage continued stimulation through D_2 or I is superimposed on the uninterrupted D_1 stimulation, a large tetanic contraction occurs, which however returns to the baseline much faster than it would have had the muscle been stimulated solely through these electrodes. If the stimulation of D_2 or I is interrupted at this moment, while D_1 stimulation continues, the response to D_2 or I stimulation recovers.

When continued stimulation is applied through D_2 , the responses both to D_1 and I stimulation appear within a short time and are present throughout the development of fatigue.

The continued stimulation through D_1 is

accompanied by an increase in muscle resistance, as indicated by the oscilloscope studies. This increased resistance is related in time of appearance to the return of response to indirect stimulation. The effect of D_2 stimulation was less marked. Investigations by means of crossed D_1 and D_2 electrodes revealed that the difference between continued D_1 and D_2 stimulation is due to the lower D_1 electrode inserted into the tendon.

The results described above could be reproduced in all experiments with only one exception. Experiments on cats were confirmatory. It appears doubtful whether the diminishing response to continued direct stimulation may be regarded as a simple fatigue phenomenon. Rather it appears more likely to be due to some kind of rectification or polarization in the tissues, however, not to polarization of the electrodes themselves.

The more intimate mechanism of these effects is being investigated. The effects themselves appear to have some practical significance in connection with the therapeutic use of alternating currents in muscle stimulation. They may also be of importance in relation to fundamental aspects of excitability and fatigue in striated muscle.

15236

"Folic Acid" Active Compounds in the Nutrition of the Monkey *

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Two years ago we reported¹ that the norite eluate concentrate of Hutchings *et al.*,² a rich source of "folic acid," was active in producing normal growth and preventing leucopenia in monkeys given a synthetic ration containing all the B vitamins available in pure form. Hutchings *et al.*³ had shown previously that this concentrate contained factors necessary

for the normal development of chicks. More recently Briggs *et al.*⁴ concluded that the norite eluate contains at least 3 factors: folic acid necessary for the growth of *Lactobacillus casei*, vitamin B_{10} , necessary for normal feathering, and vitamin B_{11} , necessary for normal growth in chicks. They⁵ further demonstrated that fractions high in vitamin B_{10} and

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Merck and Co., Rahway, N. J., for the crystalline vitamins; to Wilson Laboratories, Chicago, Ill., for the various liver preparations; to Dr. J. J. Paffner of Puze, Davis and Co., Detroit, Mich., for the crystalline B_c and B_c conjugate; to Dr. B. L.

B_{12} activity but low in folic acid can promote excellent growth and feathering in the chick. Pfäfer *et al.*¹ isolated a crystalline compound from liver which they called vitamin B_{12} and found that it has both growth-promoting and nemoplastic properties for the chick. Since vitamin B_{12} also has growth-promoting properties for *Lactobacillus casei* these workers suggested that vitamin B_{12} Hutchings norite eluate factor and Williams folic acid² are probably identical. Binkley and coworkers³ isolated from yeast a B_{12} conjugate which has vitamin B_{12} activity for the chick but no activity for the bacterium. However, upon enzymatic digestion the B_{12} conjugate becomes active as a source of B_{12} for the *Lactobacillus casei*. Day and coworkers⁴ reported that highly purified *Lactobacillus casei* factor was effective in treating vitamin M-deficient monkeys. In this paper we wish to report the relative effectiveness of vitamin B_{12} and B_{12} concentrates vitamin B_{12} vitamin B_{12} con-

jugate and the synthetic *Lactobacillus casei* factor when used in place of the norite eluate concentrate in our purified rations for the monkey.

Experimental The method of handling the monkeys has been described previously.¹⁰ The M-2 basal diet consisting of sucrose 73 Labco casein 18 salts IV + corn oil 2 cod liver oil 3 was fed *ad libitum* and supplemented with adequate amounts of ascorbic acid thiamine riboflavin nicotinic acid pyridoxine, choline pantothenic acid inositol, *p*-aminobenzoic acid and biotin.

A liver fraction high in B_{12} and B_{12} activity but low in folic acid activity was prepared according to the directions of Briggs *et al.*⁵ In brief this method consists of dialysing a concentrated superfiltrate⁶ in a cellophane bag against cold water for 96 hours. The residue is saved and placed in a Wisking cellulose casing and dialysed against hot distilled water. The dialysate is saved and concentrated so that 1 cc of the final solution is equivalent to 10 g of the original solubilized liver extract (fraction L).

The dialysate was assayed for B_{12} and B_{12} activity using chicks and for folic acid using the microbiological method. Using as a standard (100%) the growth and feathering of chicks fed a ration containing 2% solubilized liver (fraction L) one cc of the dialysate showed 95% B_{12} and B_{12} activity. Assay with *Streptococcus lactis* showed that 1 cc of the dialysate contained less than 10% folic acid using crystalline vitamin B_{12} as the standard.

States with B_{12} and B_{12} Concentrates Three monkeys No 217 218 and 219 were placed on the basal ration and given daily 1 cc of the concentrated dialysate. The monkeys grew at a slow rate for 8 weeks after which they began to lose weight. The supplement was then increased to 2 cc per day equivalent to 20 g of the original solubilized liver. This too proved to be inadequate and within two weeks the monkeys became cachectic and developed anorexia. Monkeys 217 and 219 were

Hutchings of Lederle Laboratories, Inc., Pearl River, N.Y. for the synthetic *Lactobacillus casei* factor to Mrs. E. A. Jones for assistance in the differential counts to Mr. T. D. Luckey for the B_{12} and B_{12} assays and to Mrs. Lillian Plummer for the folic acid assays.

¹ Wasman, H. A. and Ehrenheim, C. A. *J. Nutrition* 1947 26 261.

² Hutchings, B. L., Bolones, N. and Peterson, W. H. *J. Biol. Chem.* 1941 141 521.

³ Hutchings, B. L., Bolones, N., Hogsted, D. M., Ehrenheim, C. A. and Peterson, W. H. *J. Biol. Chem.* 1941 140 681.

⁴ Briggs, G. M., Luckey, T. D., Ehrenheim, C. A. and Hart, E. B. *J. Biol. Chem.* 1943 148 163.

⁵ Briggs, G. M., Luckey, T. D., Ehrenheim, C. A. and Hart, E. B. *J. Biol. Chem.* 1945 158 303.

⁶ Paffen, J. I., Butler, S. B., Bloom, E. S., Brown, R. A., Boyd, O. D., Emmett, A. D., Hegan, A. G. and O'Dell, P. L. *Science* 1943 97 404.

⁷ McColl, H. K., Smith, E. E. and Williams, P. I. *J. Am. Chem. Soc.* 1941 63 228.

⁸ Butler, S. B., Boyd, O. D., Bloom, E. S., Brown, R. A., Callans, D. G., Campbell, C. J., Emmett, A. D. and Paffen, J. I. *Science* 1944 100 37.

⁹ Day, P. L., Mims, V., Toller, I. B., Smith, E. L., Hutchings, B. L. and Bolones, N. H. *J. Biol. Chem.* 1945 157 423.

¹⁰ Wasman, H. A., Rasmussen, A. F., Jr., Ehrenheim, C. A. and Clark, P. F. *J. Nutrition* 1947 26 115.

given 2 cc of the crude norite eluate concentrate daily equivalent to 10 g of the original solubilized liver in place of the dialysate, and within a week they began to gain weight rapidly, and the cachexia soon disappeared. Four weeks later the norite eluate concentrate level was reduced to 1 cc per day equivalent to 5 g solubilized liver, and the monkeys continued to grow at the same rate.

Monkey 218 developed a severe diarrhea and refused to eat the ration. Since folic acid-deficient monkeys occasionally develop dysentery, this monkey was given 1 g of sulfasuxidine per day. In addition the M-2 basal was supplemented with 3% whole liver powder which was fed together with the sulfasuxidine by stomach tube. After a week the diarrhea disappeared, and the monkey regained its appetite so that the tube feeding was no longer necessary. Shortly thereafter a sharp increase in weight occurred, and the monkey continued to gain at a rapid rate. Apparently a concentrate showing high vitamin B₁₀ and B₁₁ activity is inadequate as a source of the essential factor for the monkey. On the other hand the crude norite eluate concentrate carries the active factor since it caused a resumption of growth when fed to the deficient monkeys.

Studies with Crystalline B₉, B₉ Conjugate, and Synthetic Lactobacillus casei Factor. The same monkeys (No 217, 218, and 219) were used in this study and were returned to the basal ration after having been on the folic acid complete ration for 3 months. In the case of monkeys 217 and 219 the norite eluate concentrate was discontinued while the whole liver powder was removed from the diet of monkey 218. After 10 weeks on the basal ration the growth rates of these 3 monkeys decreased considerably. At this point monkey 217 had a white blood cell count of 8,000 per cmm, monkey 218 5,000 per cmm, and monkey 219 5,000 per cmm. These low counts and the reduced growth rates were good indications that a characteristic "folic acid" deficiency existed.¹ A week later the animals began to lose weight, and accordingly therapy with B₉ and B₉ conjugate was instituted. 100 γ of vitamin B₉ per day was added to the ration of monkey 217, and 200 γ B₉ conjugate to the ration of monkeys 218 and 219. White

blood cell counts were made 3 times a week.

Preliminary results showed that the free vitamin B₉ caused a more rapid response than an equivalent amount of the conjugate. However, it soon became apparent that a complicated deficiency existed in two of the monkeys and so individual case histories are given for purposes of clarification.

No 219. After 2 weeks of supplementation with 200 γ B₉ conjugate the white blood cell count rose from 5,000 to 11,000 per cmm and the animal maintained its weight. During the next week the monkey showed a small increase in weight. The B₉ conjugate level was then increased to 300 γ per day, but this change had no additional effect either on the white blood cell level or weight. Two weeks later the monkey started to lose weight but the white blood cell count did not fall. 100 γ B₉ per day was substituted for the B₉ conjugate but the monkey continued to lose weight during the next 2 weeks. Substituting 150 γ of the synthetic *Lactobacillus casei* factor for the B₉ for one week also proved ineffective in checking the decline in weight. Hemoglobin determinations showed that the monkey was anemic, the values averaging 8 g of hemoglobin per 100 cc of blood. Differential white cell counts indicated a definite reversal in the neutrophile-lymphocyte ratio. These changes are typical symptoms of a deficiency of the monkey anti-anemia factor.^{11,12} Since the leucocyte count was 16,000 per cmm it seemed unlikely that a concomitant folic acid deficiency existed. Whole liver powder, a good source of the monkey anti-anemia factor¹³ was then fed at a level of 3% of the ration. The monkey rapidly increased in weight, gaining 600 g in one week, and the hemoglobin level rose to 12.2 g per hundred cc. The neutrophile-lymphocyte ratio returned to normal within 2 weeks. Evidently the B₉ conjugate, B₉, and the synthetic *Lactobacillus casei* factor were effective in combatting the

¹¹ Cooperman, J. M., Worsman, H. A., McColl, K. B., and Elvehjem, C. A., *J. Nutrition*, 1945, **30**, 45.

¹² McColl, K. B., Worsman, H. A., Elvehjem, C. A., and Jones, E. S., *J. Nutrition*, in press.

¹³ Cooperman, J. M., McColl, K. B., and Elvehjem, C. A., *Science*, 1945, **102**, 645.

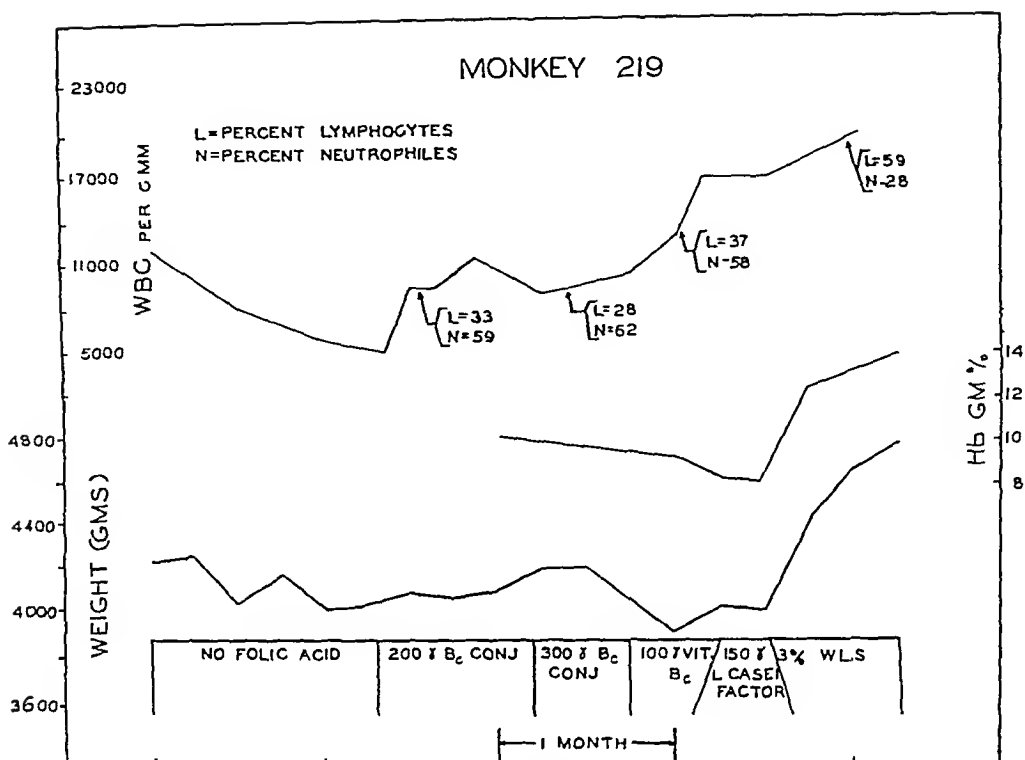


FIG 1

The effect of B_c conjugate, vitamin B_c, the synthetic *L. casei* factor and whole liver powder on the growth leucocytes, and hemoglobin of a monkey with a complicated folic acid deficiency. The levels of the various supplements were fed daily for the period of time indicated on the graph.

γ B_c conj = μg of B_c conjugate

γ Vit B_c = μg of vitamin B_c

γ *L. casei* factor = μg of synthetic *Lactobacillus casei* factor

W L S = whole liver substance (whole liver powder)

folic acid deficiency but whole liver powder was necessary in addition for the monkey to recover from the monkey anti-anemia factor deficiency which was precipitated. The data are summarized in Fig 1.

No 218 The ration of this animal was supplemented with 200 γ B_c conjugate. During the next 10 days the monkey continued to lose weight, and the leucocyte count did not rise significantly. Hemoglobin determinations showed that the animal was not anemic since the values were in the range of 14.3 g per hundred cc. The monkey was then given 100 γ B_c per day instead of the conjugate, and within a week the monkey increased in weight and the leucocyte count increased to

18,000 per cmm. The weight gain continued as did the rise in leucocytes, and after 4 weeks the synthetic *Lactobacillus casei* factor was substituted for the B_c at a level of 100 γ per day. The weight increase continued at the same rate and the leucocyte count eventually reached 26,000 per cmm. Differential leucocyte counts showed a normal neutrophile-lymphocyte ratio. In this case there appeared to be no complicating deficiencies. The B_c conjugate at a level of 200 γ per day proved to be insufficient in correcting the folic acid deficiency; on the other hand 100 γ B_c per day was quite effective as was the synthetic *Lactobacillus casei* factor. Since this monkey had been given a ration containing 3% whole

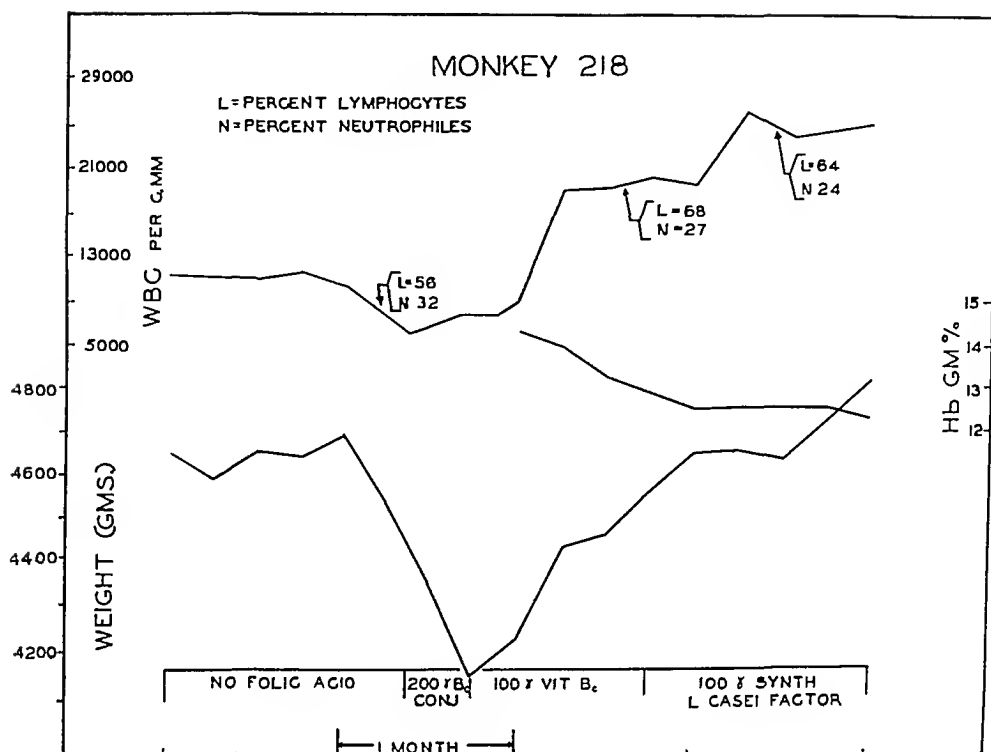


FIG 2

The effect of B₁₂ conjugate, vitamin B₁₂, and the synthetic *L casei* factor on the growth, leucocytes, and hemoglobin of a monkey with an uncomplicated folic acid deficiency

The levels of the various supplements were fed daily for the period of time indicated on the graph

γ B₁₂ conj = μg of B₁₂ conjugate

γ Vit B₁₂ = μg of vitamin B₁₂

γ *L casei* factor = μg of synthetic *Lactobacillus casei* factor

liver powder for 3 months before it was placed on the M-2 basal, it is very likely that it had an adequate store of the monkey anti-anemia factor and developed an uncomplicated folic acid deficiency. The data for this animal are summarized in Fig 2

No 217 The weight loss in this monkey was arrested after the animal received 100 γ of B₁₂ per day for 1 week and the leucocyte count rose to 13,000 per cmm. During the next 3 weeks the leucocytes remained at a level of 14,000 per cmm but there was only a slight increase in weight. The level of B₁₂ was increased to 150 γ per day and during the next 10 days the weight increased at a slightly greater rate. At this time the leucocyte count was 20,000 per cmm. 150 γ of the synthetic *Lactobacillus casei* factor per day

was then substituted for the B₁₂ and the monkey continued to grow at the same rate and maintained its leucocyte count. Hemoglobin values ranged between 11 and 12 g per 100 cc for this monkey and differential counts showed a reversed neutrophile-lymphocyte ratio. These together with the poor growth rate indicated the existence of a complicating deficiency of the monkey anti-anemia factor. The leucocyte count on the other hand responded to the B₁₂ and *Lactobacillus casei* factor.

Discussion The crude norite eluate concentrate has been used in our earlier work as a source of folic acid in our purified monkey ration. Since it is desirable to substitute as soon as possible, crystalline compounds for crude concentrates in nutritional studies, folic

acid-like compounds were tested for activity Vitamin B₁₀ and B₁₁ concentrates which are prepared from the crude norite eluate apparently do not possess activity for the monkey but do contain compounds which are available for the chick Crystalline vitamin B_c or the synthetic *Lactobacillus casei* factor can replace the norite eluate concentrate in the ration of the monkey each possessing about equal activity Evidently the B_c conjugate is less active than either of these two compounds

A deficiency in the monkey anti-anemia factor complicated the folic acid deficiency, similar conditions have been observed in riboflavin,¹¹ B₆ and pantothenic acid¹² deficiency in the monkey This deficiency is characterized by a suboptimal hemoglobin level, lack of growth and a reversal in the neutrophile-lymphocyte ratio In the normal monkey the lymphocytes constitute 60-70% of the total leucocytes and the neutrophiles 25-35% However, in monkeys deficient in the monkey anti-anemia factor the ratio is reversed and the neutrophiles rise to 60-70% of the total leucocytes and the lymphocytes fall to 25-35% while the total leucocyte count is not appreciably reduced There is, therefore, an actual increase in the number of neutrophiles and a decrease in the number of lymphocytes Therapy with whole liver corrects this reversal, and produces a normal hemoglobin level and a resumption of growth

The folic acid deficiency evidently precipitated a deficiency of this factor in the monkeys

which had never received a ration containing whole liver Thus in monkey 219 the low leucocyte count due to folic acid deficiency was corrected by vitamin B_c but whole liver was necessary to correct the neutrophile-lymphocyte ratio reversal, the low hemoglobin levels and the loss in weight On the other hand monkey 218 was fed 3% whole liver powder in the ration for 3 months and consequently had an adequate store of this factor when the folic acid deficiency developed This animal showed the best response to the various crystalline supplements since the folic acid deficiency was uncomplicated Monkey 217, like monkey 219, showed a good leucocyte response to therapeutic doses of vitamin B_c The weight response on the other hand was poor and blood studies showed that a dyscrasia existed typical of a deficiency of the monkey anti-anemia factor

Summary Vitamin B₁₀ and B₁₁ concentrates were inactive as a source of folic acid for the monkey

Crystalline vitamin B_c and the synthetic *Lactobacillus casei* factor were active as a source of folic acid for the monkey when fed at a level of 100 γ per day The B_c conjugate had less activity when fed at levels of 200 and 300 γ per day

Folic acid deficiency precipitates a deficiency for the monkey anti-anemia factor which is characterized by lack of growth, suboptimal hemoglobin levels and a reversal in the lymphocyte-neutrophile ratio

15237

A Differential, Microbiological Assay for O-Heterobiotin *

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O-Heterobiotin (an analog of biotin in which the sulfur atom has been replaced by an oxygen atom) has biotin-like activity for *L casei*,^{1,2,3} *S cerevisiae*,^{1,2,3} *R trifolii*,⁴

L arabinosus,^{3,5} chicks,³ and for counteracting egg white toxicity in rats^{3,5} Hofmann *et al* have developed an assay for O-heterobiotin with *S cerevisiae* after the biotin in the sample

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has been destroyed by permanganate oxidation⁴ In an attempt to develop a differential microbiological assay for O-heterobiotin we have tested its activity for representative microorganisms from several different genera The results with *L. arabinosus* 17-5 and *S. faecalis* R are presented here

Experimental The general procedures used and the preparation of the basal medium constituents are similar to those used in the assay of folic acid⁶ A 24-hour-old inoculum of each microorganism is centrifuged and resuspended separately in sterile saline solution One drop of each suspension is inoculated separately into duplicate sterilized tubes which contain 5 ml of basal medium (Table I), the sample, and distilled water to give a total volume of 10 ml Standard curves with biotin and O-heterobiotin are run simultaneously with graded levels of the sample The tubes are incubated at 30° for 16-20 hours and the growth is measured with the Evelyn photoelectric colorimeter

Since the activity of O-heterobiotin for *L. arabinosus* may vary from one assay to another, a factor relating the activity of O-heterobiotin to that of biotin must be obtained for each assay An average of 3-5 ratios obtained from different sections of the standard curves gives a reliable activity factor since the ratios are constant within each assay

The biotin content of a sample may be determined directly from the standard biotin curve obtained with *S. faecalis* The O-heterobiotin content may be calculated by multi-

plied by the crystalline vitamins and Dr S H Rubin and Dr J A Aeschlimann of Hoffmann-LaRoche, Inc, Nutley, N J, for the O heterobiotin

¹ Duschinsky, R, Dolan, L A, Flower, D, and Rubin, S H, *Arch Biochem*, 1945, **6**, 480

² Pilgrim, F J, Axelrod, A E, Winnick, T, and Hoffmann K, *Science*, 1945, **102**, 35

³ Hoffmann, K, McCoy, R H, Felton, J R, Axelrod, A E, and Pilgrim, F J, *Arch Biochem*, 1945, **7**, 393

⁴ Hoffmann, K, and Winnick, T, *J Biol Chem*, 1945, **160**, 449

⁵ Rubin, S H, Flower, D, Rosen, F, and Dreker, L, *Arch Biochem*, 1945, **8**, 79

⁶ Luckey, T D, Buggs, G M, Jr, and Elvehjem, C A, *J Biol Chem*, 1944, **152**, 157

TABLE I
Medium for O Heterobiotin Determination

Constituent	Amt for 10 tubes (100 cc of complete medium or 50 cc basal)
Casein	0.5 g
Tryptophane	0.02
Cystine	0.01
Glucose	1.0
Na acetate	0.4
K ₂ HPO ₄	0.5
Spermin's salts B	0.5 cc
Adenine SO ₄	1.0 mg
Guanine HCl	1.0
Xanthine	1.0
Uric acid	1.0
Thiamin HCl	50 γ
Riboflavin	50
Nicotinic acid	50
Pyridoxine HCl	50
Pyridoxal HCl	0.2
α Pyrimin*	0.2
Biotin	0.00
Synthetic folic acid†	0.2
Ca pantothenate	50
p Aminobenzoic acid	10
Water	50 cc
pH	6.8

* 5 Pyridoxine acid, or the lactone of 2 methyl 3 hydroxy 4 hydroxy methyl 5 carboxypyridine are other names for this compound obtained from Meier and Co through the courtesy of Dr Karl Folkers

† Obtained through the courtesy of Dr B L Hutchings at Lederle Laboratories, Inc

plying the activity factor for O-heterobiotin by the difference between the apparent biotin content as measured with *L. arabinosus* and the biotin content obtained with *S. faecalis* These calculations may be summarized

$O = k (Bt - Bt_0)$, where
 O = O heterobiotin content of the sample,
 k = activity factor for O heterobiotin compared to biotin with *L. arabinosus*, or

amount of O heterobiotin needed to produce x growth

$=$ amount of biotin needed to produce x growth

Bt_0 = apparent biotin content as determined with *L. arabinosus*, and

Bt = true biotin content obtained with *S. faecalis*

The following procedure is used to liberate bound biotin Add one gram of sample to 25 ml of 2N/H₂SO₄, autoclave at 122° for 2 hours, neutralize with 4N/NaK(OH)₂ (a mixture of sodium and potassium hydroxide is used to keep a balance of these ions in the medium), filter and dilute as needed When a

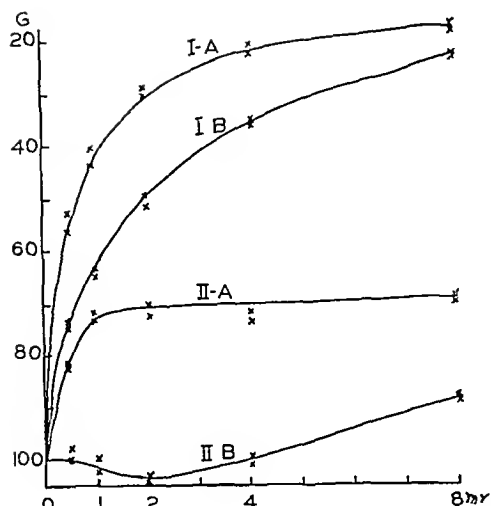


Fig 1

Standard curves with biotin (A) and O heterobiotin (B) obtained with *L. arabinosus* (I) and *S. faecalis* (II)

* G = Galvanometer reading with inoculated blank set at 100

TABLE II
Effect of pH on the Activity of *d,l* O Heterobiotin

pH	% activity compared to <i>d</i> biotin		Tubes used for each value
	<i>Sf</i> *	<i>La</i> †	
6.6	0.0	33	20
6.5	1.7	44	40
7.0	2.5	39	40
7.3	7.5	29	20

* *Sf* = *Streptococcus faecalis*

† *La* = *Lactobacillus arabinosus*

small amount of O-heterobiotin was hydrolyzed its activity for *L. arabinosus* decreased slightly

Throughout this work *d*-biotin has been compared to *d,l*-O-heterobiotin. If only the *d*-form of O-heterobiotin is active, the values given here should be increased correspondingly.

Results A typical set of curves for the assay of O-heterobiotin (Fig 1) indicates that its activity relative to biotin is constant throughout the curves obtained with *L. arabinosus*. Low levels of O-heterobiotin are somewhat inhibitory for *S. faecalis* while higher levels are about 2% active. The activity of O-heterobiotin for *S. faecalis* may be disregarded since any amount of O-heterobiotin

which will produce appreciable growth for this organism (more than 8mγ) gives maximum growth for *L. arabinosus*.

In an attempt to find why the activity factor for O-heterobiotin varied with different assays, standard curves were run at various pH levels. The results (Table II) indicate that the activity of O-heterobiotin relative to biotin for *S. faecalis* increases as the pH is increased from 6.6 to 7.3, while the activity of O-heterobiotin relative to biotin for *L. arabinosus* is greatest at pH 6.8. Thus, pH 6.8, which was originally used, gives the greatest differentiation.

In order to test the effect of biotin upon the activity of O-heterobiotin graded levels of O-heterobiotin were added to tubes containing a known amount of biotin. The amount of O-heterobiotin found corresponded well with the amount added (Table III). The amount of biotin found in tubes containing large amounts of O-heterobiotin was greater than the amount of biotin added. This may be due to an increased sensitivity in both bacteria for O-heterobiotin in the presence of biotin.

When certain natural materials were analyzed for O-heterobiotin the results (Table IV) indicated that if this compound occurs naturally its extent is limited. When O-heterobiotin was added to fresh kidney, after acid hydrolysis, the biotin content as measured with *S. faecalis* was identical to the value obtained for kidney without the added O-heterobiotin. The apparent biotin content, as measured with *L. arabinosus* was higher. When the values obtained were applied in the above formula, the recovery of O-heterobiotin was very satisfactory (Table IV, No 8).

Discussion The name O-heterobiotin is used in this paper in conformity with the first common name to appear in the literature¹ rather than the later name oxybiotin² since the latter does not definitely distinguish between this compound and such compounds as biotin sulfoxide and biotin sulfone.

Since O-heterobiotin does not seem to occur naturally, biotin values previously obtained are probably not invalidated by this new compound. However, there does seem to exist naturally a compound in chicken muscle and fresh lamb kidney which has more biotin

TABLE III
Recovery of O Heterobiotin

Biotin per tube, $\mu\gamma$	O heterobiotin per tube, $\mu\gamma$	Biotin found, [*] $\mu\gamma$	O heterobiotin found, [†] $\mu\gamma$	% recovery of O heterobiotin
0.5	0.5	0.5	0.48	96
0.5	1	0.6	1.14	114
0.5	1	0.6	0.96	96
0.5	2	0.75	2.10	105
0.5	4	0.75	4.35	109

* *S. faecalis* value

† (*L. a.* value—*S. f.* value) \times factor = O heterobiotin

TABLE IV
O Heterobiotin Content of Naturally Occurring Materials

Material	γ "Biotin"/g		γ O heterobiotin/g
	<i>S. faecalis</i>	<i>L. arabinosus</i>	
1 Fresh beef liver	1.2	1.1	0.0
2 Fresh chuck liver	0.90	0.88	0.0
3 Fresh chuck leg muscle	0.15	0.02	0.0
4 Difco yeast extract	3.3	3.3	0.0
5 Grass juice powder	0.40	0.39	0.0
6 Vitab	1.0	0.93	0.0
7 Fresh lamb kidney	0.90	0.66	0.0
8 Fresh lamb kidney + 1 γ O heterobiotin	0.90	1.61	1.0

activity for *S. faecalis* than for *L. arabinosus*. When O-heterobiotin is fed or injected into animals, it might be expected to occur to some extent in the tissues.

Summary A differential microbiological assay for O-heterobiotin with *S. faecalis* R and

L. arabinosus 17-5 has been developed. Assays of several natural materials indicated that O-heterobiotin does not occur generally, but that a metabolite does exist in animal tissues which has biotin activity for *S. faecalis* and not for *L. arabinosus*.

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15238 P

Sodium Salicylate Inhibiting Anti-Rh Immunization in Animals

F HOMBURGER (Introduced by G R Minot)

From the Thorndike Memorial Laboratory, Second and Fourth Medical Services (Harvard), Boston City Hospital, and the Department of Medicine, Harvard Medical School, Boston

The inhibition of anti-RH agglutinins by ethylmercuri-thiosalicylate *in vitro* has been shown by Waller¹ and sodium salicylate is known to impair the formation of antipneumococcic immune antibodies in rabbits.² The present preliminary study suggests that sodium salicylate inhibits the formation of anti-Rh agglutinins in guinea pigs and in rabbits.

Experimental Procedure Forty guinea pigs and 20 rabbits were used. All animals were immunized by the injection of red blood cells from rhesus monkeys. Guinea pigs received intraperitoneal injections of 1 cc of washed rhesus cells suspended in saline on the third and on the eighth day of the experiment. Rabbits were given intravenous injections of 0.01 cc of citrated rhesus blood in 1 cc of saline on the 4th, 5th, 6th, 11th, 12th and 13th days of the experiment. Half of the number of the animals received 0.16 g of sodium salicylate per kg of body weight daily by stomach tube (guinea pigs) or by subcutaneous injection (rabbits) starting the first day of the experiment and continued up to 48 hours be-

fore the blood was examined, 7 days after the last injection of rhesus blood. Anti-Rh agglutinins were looked for by the method of Landsteiner and Wiener³ in the case of the guinea pigs⁴ and by a modification of Diamond's⁵ method, using human albumin[†] in the case of the rabbits. Negative readings were confirmed by microscopic examination. Serum salicylate levels were measured by the method used by Coburn.⁵

Results and Discussion Of 21 animals[‡]

³ Landsteiner, K, and Wiener, A S, *J Exp Med*, 1941, **74**, 309.

⁴ Diamond, L K, and Denton, R L, *J Lab and Clin Med*, 1945, **10**, 821.

* Some of these sera were examined by Dr L K Diamond whose cooperation is gratefully acknowledged.

† The albumin used was a sample prepared by the Harvard Fractionation Laboratory and obtained through the courtesy of Prof E J Cohn.

⁵ Coburn, A F, *Bull Johns Hopkins Hosp*, 1943, **73**, 435.

‡ An epizootic described elsewhere⁶ destroyed 9 of the guinea pigs and 12 rabbits died from aspiration pneumonia.

⁶ Homburger, F, Wilcox, C, Barnes, M W, and Finland, M, *Science* 1945, **102**, 449.

¹ Waller, K, *Am J Clin Path*, 1944, **8**, 116.

² Swift, H F, *J Exp Med*, 1922, **30**, 735.

that had not received any sodium salicylate, 15 showed anti-Rh agglutinins following the injection of rhesus blood. In the case of the rabbits these were stronger which suggests that the methods used on these animals were better suited for such experiments.

Of 18 animals which had received sodium salicylate during the period of immunization only 2 showed microscopic agglutination of Rh positive cells.

No free salicyl radical was found in any of the sera tested which suggests that *in vivo* salicylate inhibits the formation of agglutinins rather than their effect in the plasma.

These findings call for confirmation and salicylate prophylaxis for erythroblastosis fetalis is not suggested here. On the contrary, the toxicity of this drug is again emphasized. However, these experiments intimate the possibility of a chemotherapeutic approach to the subject of iso-immunization.

Summary In guinea pigs and in rabbits the formation of anti-Rh agglutinins following the injection of rhesus monkey blood cells appears to be diminished when sodium salicylate has been administered 3 days prior to and during the period of immunization.

† Homburg, F. *Am J Med Sci* in press

15239 P

Biochemical Changes Following Poisoning of Rats by Alphanaphthylthiourea

K. P. DuBois, L. W. Holm, and W. L. Doyle

From the University of Chicago Toxicity Laboratory *

Alphanaphthylthiourea (ANTU) has been proposed as a rodenticide because of its effectiveness against Norway rats. Richter¹ has shown that poisoning by this compound is characterized by (a) development of intense pulmonary edema and pleural effusion, (b) marked resistance of herbivores in comparison to carnivores, (c) greater resistance of younger animals, (d) a rapid development of tolerance after repeated sublethal doses. These factors are individually significant in the action of many drugs. The present study indicates that ANTU profoundly alters the carbohydrate metabolism in poisoned animals and that the administration of cysteine counteracts these effects in rats.

Experimental Purina Chow was used as diet for adult albino rats. ANTU was administered intraperitoneally in propylene

glycol (5 mg ANTU per ml solvent). The solvent alone produced no observable effects. Under these conditions the LD-50 was approximately 3.0 mg per kilo. A dose of 10 mg per kg killed all rats between 6 and 24 hours after injection. Glucose was determined by the method of Folin and Malmros² and glycogen according to Good *et al.*³ Our initial experiments consisted blood glucose determinations on rats prior to and 2.5 hours after injection of 10 mg per kg of ANTU. In 6 rats the average normal blood glucose rose from 68 ± 2 mg % to an average of 173 ± 18 mg % in 2.5 hours after poisoning. It was later observed that this initial hyperglycemia was followed by hypoglycemia which might be due to depletion of liver glycogen. Eight rats were fasted for 7 hours after which time 4 received 10 mg per kg of ANTU intraperitoneally. Food was withheld from both groups for another 8 hours after which

* This work was carried out under contract with the Medical Division of the Chemical Warfare Service.

¹ Richter, C. P., *J. Am. Med. Assn.*, 1945, **129**,

- Folin, O., and Malmros, H., *J. Biol. Chem.*, 1929, **83**, 115.

³ Good, C. A., Kramer, H., and Somogyi, M., *J. Biol. Chem.*, 1933, **100**, 484.

TABLE I
Effect of ANTU on Synthesis of Liver Glycogen

Glucose schedule	Controls		ANTU—10 mg/kg	
	Rat No	Glycogen %	Rat No	Glycogen %
None	15	0.14	22	11
	16	0.14	23	07
0.15 g/hr for 2 hr before death	17	0.48	24	09
	18	0.28	25	12
" " " 4 " " "	19	0.95	26	03
	20	1.20		
" " " 6 " " "	21	1.20	27	10
			28	05

TABLE II
Effect of Cysteine on the Toxicity of ANTU

Group	ANTU (mg/kg)	Cysteine (mg/kg)	Mortality	% Mortality
1	10	0	5/5	100
2	10	200	2/2	100
3	10	400	3/5	60
4	10	700	1/5	20
5	10	1000	0/5	0

time all of the animals were sacrificed by decapitation. The livers were removed and frozen on dry ice. Decapitation and removal of the livers was accomplished within 1 minute to prevent glycogenolysis. Whereas the average liver glycogen values for the control animals was $1.13\% \pm 25$, that of the ANTU-poisoned group was only $0.019\% \pm 0.06$, clearly indicating a depletion of liver glycogen in rats poisoned with ANTU.

The glycogenic activity of the liver of ANTU-poisoned animals was then examined. Fourteen female rats were fasted 16 hours after which time 7 rats were given 10 mg per kg of ANTU intraperitoneally. A 10% glucose solution was injected intraperitoneally at hourly intervals into the fasted controls and the ANTU-poisoned animals. All the animals were killed 6 hours after ANTU was injected and liver glycogen was determined as previously described. The quantity of glucose injected per rat and the results of the liver glycogen determinations are given in Table I. The animals which had received 10 mg per kg of ANTU were unable to deposit liver glycogen, while controls deposited glycogen after glucose injections.

Possible methods of counteracting ANTU

poisoning are of interest not only with respect to the mechanism of action but also from the standpoint of obtaining therapeutic measures for accidental poisoning. It appeared possible that ANTU reacts with sulphhydryl groups. Cysteine was the first sulphhydryl compound tested. Rats were given 10 mg per kg of ANTU (5 mg per ml in propylene glycol) intraperitoneally. Varying amounts of cysteine (100 mg per ml in saline) were injected intraperitoneally into 5 groups of rats immediately after injection of ANTU. The results given in Table II show that cysteine, at a level of 1 g per kg effectively counteracted the lethal action of an LD-100 dose of ANTU, and partial protection was obtained by lower doses of cysteine in rats. Additional results indicate that intraperitoneal injection of cysteine is equally effective against ANTU administered by stomach tube. Protection to the same degree was not obtained in dogs receiving a lethal dose of ANTU.

Summary and Conclusions The intraperitoneal injection of 10 mg per kg of ANTU into rats caused a marked rise in blood sugar from an average value of 68 mg % and 173 mg % in 2.5 hours. Liver

glycogen values fell from a normal of 1.13% to 0.019%. ~~ANTU-poisoned animals were unable to deposit liver glycogen~~ While these experiments demonstrate a disturbance in carbohydrate metabolism, they do not prove that death from ANTU is due to these changes. In rats large doses of cysteine (1000 mg per kg) afforded protection from death against 10 mg per kg of ANTU. The relation between lung damage and impairment of glycolysis and/or respiration is under investigation.

15240

Modification of Anaphylaxis by Benadryl *

J. A. WELLS, H. C. MORRIS, AND CARL A. DRAGSTEDT

From the Department of Pharmacology, Northwestern University Medical School, Chicago, Ill

It has been recently shown that beta-dimethyl aminoethyl benzhydryl ether hydrochloride (Benadryl) inhibits the action of histamine on the bronchi and ileum of the guinea pig.¹ The subsequent demonstration that this drug is capable of reducing the severity of anaphylaxis in the guinea pig² is in accordance with the repeated demonstration that anaphylaxis in the guinea pig is due, in large part, to bronchospasm resulting from the action of histamine which is liberated from the lung.³⁻⁵

We have demonstrated that Benadryl inhibits the vasodepressor action of histamine in the anesthetized dog and have suggested that this inhibition is due to competition of

Benadryl with histamine for its site of action.^{9,10} The purpose of the present investigation was to determine the effect of Benadryl against anaphylaxis in the dog, a condition which, as in the guinea pig, is intimately connected with the release and action of histamine.¹¹⁻¹⁵

It has been shown that in fatal anaphylaxis in the dog from 3.2 to 4.7 mg of histamine per kilo (measured as acid phosphate) is liberated from the liver.¹⁶ It is also known that such doses of histamine injected intravenously in dogs produce fatal shocks.^{13,17} Therefore, preliminary to studies of anaphylaxis, a series of 8 dogs were injected intravenously with 4.5 mg of histamine acid phosphate per kilo. These animals were pretreated with a dose of Benadryl (100 mg

* This study was aided in part by a grant from the Clara A. Abbott Fund of Northwestern University Medical School.

The Benadryl used in these studies was furnished to us through the courtesy of Parke, Davis and Company.

¹ Loew, E. R., Krusei, M. E., and Moore, V., *J. Pharm. and Exp. Therap.*, 1945, **83**, 120.

² Loew, E. R., and Krusei, M. E., *Proc. Soc. Exp. Biol. and Med.*, 1945, **58**, 235.

³ Bartosch, R., Feldberg, W., and Nigle, E., *Pflüger's Arch.*, 1932, **230**, 129.

⁴ Welschstein, M., *Pflüger's Arch.*, 1932, **231**, 24.

⁵ Daly, I., Pent, S., and Schild, H., *Quart. J. Exp. Physiol.*, 1935, **25**, 32.

⁶ Ungar, G., and Piotto, J. L., *C. R. Soc. de Biol.*, 1936, **123**, 676.

⁷ Ungar, G., Piotto, J. L., and Levillain, A., *C. R. Soc. de Biol.*, 1937, **125**, 1015.

⁸ Schild, H. O., *J. Physiol.*, 1937, **90**, 34P.

⁹ Wells, J. A., and Morris, H. C., *Fed. Proc.*, 1945, **4**, 140.

¹⁰ Wells, J. A., Morris, H. C., Dragstedt, C. A., and Bull, H. B., *J. Pharm. and Exp. Therap.*, in press.

¹¹ Dragstedt, C. A., and Gebruer-Fuelnegg, E., *Am. J. Physiol.*, 1932, **102**, 512.

¹² Gebruer-Fuelnegg, E., and Dragstedt, C. A., *Am. J. Physiol.*, 1932, **102**, 520.

¹³ Dragstedt, C. A., and Merd, F. B., *J. Pharm. and Exp. Therap.*, 1936, **57**, 419.

¹⁴ Code, C. F., *Am. J. Physiol.*, 1939, **127**, 78.

¹⁵ Dragstedt, C. A., and Merd, F. B., *J. Immunol.*, 1936, **30**, 319.

¹⁶ Ojers, G., Holmes, C. A., and Dragstedt, C. A., *J. Pharm. and Exp. Therap.*, 1941, **73**, 33.

¹⁷ Dragstedt, C. A., and Merd, F. B., *Proc. Soc. Exp. Biol. and Med.*, 1935, **32**, 1435.

TABLE I
Influence of Benadryl on Anaphylaxis in the Dog

Degree of shock	No and % of animals manifesting various degrees of shock			
	(26) Non treated controls		(22) Benadryl (10 mg/kilo)	
	No	%	No	%
0	8	30.8	3	13.6
+	2	7.7	4	18.2
++	7	26.9	13	59.1
+++	0	0.0	2	9.0
++++	9	34.6	0	0.0

per kilo i v) which previous experience has shown to produce nearly maximum histamine antagonism. All of these animals had severe shocks, but none of them died. This experiment suggests that if histamine is the primary toxic factor in anaphylaxis, then no animals pretreated with 10.0 mg per kilo of Benadryl should die from anaphylaxis.

Forty-eight dogs were sensitized to horse serum by injecting 5 cc intravenously and 5.0 cc subcutaneously. From 13 to 15 days later these animals were anesthetized with sodium barbital and prepared for recording the carotid blood pressure. All animals were given a shocking dose of 10 cc of horse serum intravenously. In 22 of these animals, prior to the injection of horse serum, Benadryl (10 mg per kilo) was slowly injected intravenously.

The fall in blood pressure occurring as a consequence of anaphylaxis was roughly quantitated as follows: 0 = no fall in blood pressure, + = fall in blood pressure, but not to shock level (*i.e.* 35-45 mm of mercury), ++ = fall in blood pressure to shock level, but with recovery beginning within 30 min, +++ = fall in blood pressure to shock level with no recovery within 30 min, ++++ = fall in blood pressure to shock level with death within 30 min.

Comparison of the 2 groups of animals (Table I) reveals that in contrast to a 34.6% mortality in the untreated series, no dogs died of anaphylaxis when they had been previously treated with 10 mg per kilo of Benadryl. It is apparent from the table that all evidence of anaphylactic vasodepression is not removed by Benadryl. This is not surprising since we have shown that maximally

effective doses of this drug do not completely abolish the actions of injected histamine in the dog. Therefore, assuming histamine to be the sole toxic factor in anaphylaxis in the dog, Benadryl would not be expected to completely abolish anaphylaxis.

It is not inconceivable that Benadryl modifies anaphylaxis in the dog by some action other than an antagonism of the histamine which is released. One obvious possibility is that it prevents the release of histamine. This possibility was investigated in the following way. Two dogs which had been previously sensitized to horse serum were shocked following the administration of 3.0 mg/kilo of Benadryl. At the point at which their respective blood pressures reached shock level a sample of their blood was drawn, heparinized and centrifuged. The serum from these dogs was then assayed by comparison with standard solutions of histamine acid phosphate on the blood pressure of the atropinized anesthetized cat. The outcome of the shock in the two animals was +++ and ++ respectively. The dog showing +++ shock was shown to have the equivalent of over 10 mcgm of histamine acid phosphate per cc of serum. The dog having ++ shock was shown to have the equivalent of 1 mcgm of histamine acid phosphate per cc of serum. These are amounts of histamine which are encountered in substantial anaphylactic shocks in the absence of Benadryl and indicate that Benadryl does not materially interfere with the release of histamine in anaphylactic shock in the dog.

Summary The intravenous injection of Benadryl (10 mg per kilo) into horse serum sensitized dogs, prior to the reinjection of

glycogen values fell from a normal of 1.13% to 0.019%. ~~ANTU-poisoned animals were unable to deposit liver glycogen.~~ While these experiments demonstrate a disturbance in carbohydrate metabolism, they do not prove that death from ANTU is due to these

changes. In rats large doses of cysteine (1000 mg per kg) afforded ~~protection from death against 10 mg per kg of ANTU.~~ The relation between lung damage and impairment of glycolysis and/or respiration is under investigation.

15240

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¹ Loew, E. R., Kruser, M. E., and Moore, V., *J. Pharm. and Exp. Therap.*, 1945, **83**, 120.

² Loew, E. R., and Kruser, M. E., *Proc. Soc. Exp. Biol. and Med.*, 1945, **58**, 235.

³ Bartosch, R., Feldberg, W., and Nagle, E., *Pflüger's Arch.*, 1932, **230**, 129.

⁴ Wachstein, M., *Pflüger's Arch.*, 1932, **231**, 24.

⁵ Daly, I., Pert, S., and Schild, H., *Quart. J. Exp. Physiol.*, 1935, **25**, 32.

⁶ Ungar, G., and Purott, J. L., *C. R. Soc. de Biol.*, 1936, **123**, 676.

⁷ Ungar, G., Pariotti, J. L., and Levillain, A., *C. R. Soc. de Biol.*, 1937, **125**, 1015.

⁸ Schild, H. O., *J. Physiol.*, 1937, **90**, 34P.

⁹ Wells, J. A., and Morris, H. C., *Gen. Proc.*, 1945, **4**, 140.

¹⁰ Wells, J. A., Morris, H. C., Dragstedt, Carl A., and Bull, H. B., *J. Pharm. and Exp. Therap.*, in press.

¹¹ Dragstedt, C. A., and Gebruer Fuelnegg, E., *Am. J. Physiol.*, 1932, **102**, 512.

¹² Gebruer Fuelnegg, E., and Dragstedt, C. A., *Am. J. Physiol.*, 1932, **102**, 520.

¹³ Dragstedt, C. A., and Merd, F. B., *J. Pharm. and Exp. Therap.*, 1936, **57**, 419.

¹⁴ Code, C. F., *Am. J. Physiol.*, 1939, **127**, 78.

¹⁵ Dragstedt, C. A., and Merd, F. B., *J. Immunol.*, 1936, **30**, 319.

¹⁶ Ojers, G., Holmes, C. A., and Dragstedt, C. A., *J. Pharm. and Exp. Therap.*, 1941, **73**, 33.

¹⁷ Dragstedt, C. A., and Merd, F. B., *Proc. Soc. Exp. Biol. and Med.*, 1935, **32**, 1435.

TABLE I

Tests for Virus in Aqueous Humor of Rabbit Eye All animals were injected with 0.2 ml of a 10% suspension of mouse brain infected with the Rockefeller strain of western equine encephalomyelitis virus

Exp	No	I	Rabbit No	Eye	Interval*	Fate of mice injected with dilutions			Cornea
						10-0	10-1	10-2	
I	K423	Left	Rt	60 hr		15 5 s	s s s	s s s	Opaque
			L	" "		s s s	s s s	s s s	"
	K424	L	Rt	" "		s s s	s s s	s s s	Slight cloudiness
			L	" "		s s s	s s s	s s s	" "
II	K433	L	Rt	24 hr		2 2 2	2 2 2	2 2 2	Insufficient time
			L	" "		2 2 2	2 2 2	2 2 2	" "
	K435	L	Rt	3 days		s s s	s s s	s s s	Clear
			L	" "		s s s	s s s	s s s	"
III	K425	L	Rt	5 "		s s s	s s s	—	"
			L	" "		s s s	s s s	—	"
	K426	L	Rt	" "		3 3 3	3 3 3	—	Opaque
			L	" "		s s s	s s s	—	"
IV	K501	L	Rt	3 days		s s s	s s s	s s s	"
			L	" "		s s s	s s s	s s s	"
	K503	L	Rt	2 "		5 5 s	5 s s	s s s	"
			L	" "		3 3 3	3 3 4	4 4 4	"

* Time between inoculation into ant. chamber and aspiration of aqueous humor

† Numbers = day of death S = survived

Injection of the 12 eyes of 6 rabbits with various dilutions of the low passage strain of virus gave entirely negative results. Five of the eyes were injected with a 10% suspension of infected brain. All eyes, except 2, were injected with an inoculum which killed mice regularly in a dilution of 10^{-7} and sometimes in dilutions of 10^{-8} .

In further studies with the Rockefeller strain, negative results have been obtained in all eyes injected with virus (infected mouse brain) diluted to 1/100 or beyond. When a 10% suspension was employed, only half of the infected eyes showed the characteristic reaction. This was surprising in view of the results of the first experiment in which a 10^{-3} dilution was injected and all 3 injected eyes were positive. Unfortunately, the records showing the results of the titration of the virus used in this first experiment were lost. The later experiments were carried out after an interval of more than one year with virus that killed all mice injected with a 10^{-6} and about half those given 10^{-7} dilution. It was our recollection that the virus used in the earlier experiment killed mice in a dilution of 10^{-8} .

As shown in Table I Experiments I and III,

rabbits injected in the same experiment with the same dose of virus might differ in their reactions. Numerous aspirations and cultures of aqueous humor showed an absence of pus cells and bacteria. It was felt that the varying results indicated that the amount of virus injected approximated one 50% minimal reactive dose (analogous to "1 MLD 50").

Tests for virus in aqueous humor were carried out at 24 hours, 48 hours, 60 hours, 3 days, and 5 days with the results shown in Table I. After injection of more than 300,000 mld (for mice) of virus into the anterior chamber, the aqueous humor contained virus in considerable amount at 24 hours but little or none remained at 48 hours and later. No virus was detected in ocular tissues at 3 days in 1 experiment. Of 4 eyes tested at 5 days, one contained a moderate amount of virus. This suggests that some growth of virus may have occurred. Of the 30 rabbits used in these experiments, only 3 showed symptoms indicative of encephalitis.

An eastern strain of equine encephalomyelitis virus obtained from Dr Carl M. Eklund has been injected into the 10 eyes of 5 rabbits. All 10 eyes developed the

horse serum, reduces the severity of anaphylactic shock in these animals, there being no deaths in 22 animals as against 9 deaths in 26 controls. As Benadryl has a similar modifying effect upon the shock induced by the injection of histamine, the results of these experiments are consistent with the theory that histamine plays a significant role

in anaphylaxis in dogs. Unfortunately, Benadryl merely reduces, but does not obliterate, the vasodepressor effects of histamine and thus the present experiments with Benadryl do not permit conclusions as to whether histamine is or is not the sole vasodepressor factor in anaphylaxis in the dog.

15241

Corneal Reaction to Viruses of Equine Encephalomyelitis After Intra-Ocular Injection *

C A EVANS AND V S BOLIN

From the Department of Bacteriology and Immunology, School of Medicine, University of Minnesota

In a previous publication¹ it was reported that injection of certain viruses into the eyes of suitable animals caused specific reactions in the ocular tissues. Corneal edema and opacity were produced when the viruses of fox encephalitis, influenza, and equine encephalomyelitis were employed. It was demonstrated that virus of fox encephalitis, even in minute dosage, would infect the corneal endothelium and induce the corneal reaction in dogs, foxes and raccoons.^{2,3} The influenza virus caused an entirely similar reaction in the eyes of rabbits in spite of a complete absence of infection, by a direct toxic effect.⁴ The virus did not grow in the rabbit eye and was effective only in very large doses.

Further studies reported in this paper suggest that the corneal opacity in rabbits caused

by the viruses of western and eastern equine encephalomyelitis is analogous to the toxic reaction demonstrated for influenza virus even though these viruses may grow to a limited extent in the ocular tissues.

In our first experiment a 10^{-3} dilution of mouse brain infected with a highly virulent strain of virus of WEE (Western Equine Encephalomyelitis) was injected into the anterior chamber of one eye of each of 3 rabbits. At 48 hours all showed some cloudiness of the cornea and at 3 days the corneas of all injected eyes were opaque.

In subsequent experiments 27 rabbits have been injected with 2 different strains of western virus. One of the western strains was highly virulent and had been maintained by passage in mice for many years by Dr Carl M. Eklund, who obtained it from Dr P. K. Olitsky of the Rockefeller Institute for Medical Research. This we call the Rockefeller strain. The second strain of western virus had been isolated from a fatal human case of encephalitis by injection of brain tissue into guinea pigs. Some of the infected guinea pig brain tissue that had been kept frozen for more than a year was used to infect guinea pigs and mice, the brains of which served as the source of virus for injection into eyes. This is called the "low passage" strain of virus.

* Aided by grants from the John and Mary R. Munkle Foundation and the Graduate School of the University of Minnesota.

¹ Evans, C. A., YAMAMOTO, H. Y., and Green, R. G., *Science*, 1943, **98**, 45.

² Evans, C. A., YAMAMOTO, H. Y., and Green, R. G., *Proc. Soc. Exp. Biol. and Med.*, 1943, **53**, 183.

³ Green, R. G., Evans, C. A., and YAMAMOTO, H. Y., *Proc. Soc. Exp. Biol. and Med.*, 1943, **53**, 186.

⁴ Evans, C. A., and Rickard, D. R., *Proc. Soc. Exp. Biol. and Med.*, 1945, **58**, 73.

junction to a variable extent. Histologically, this uveitis appeared as hyperemia of the iris and ciliary body, usually with very little cellular exudate. The choroid and retina were not visibly affected after injection of either the eastern or western viruses into the anterior chamber.

Conclusion The corneal edema and opacity resulting from intra-ocular injection of the virus of western equine encephalomyelitis

in domestic rabbits occurs only in response to large amounts of virus. It appears to be analogous to the ocular response to influenza virus, which has been attributed to toxic properties of the virus. An entirely similar ocular reaction has been induced by injection of equine encephalomyelitis virus of the eastern type. Limited growth of equine encephalomyelitis virus may occur in the eye but is not a significant factor in the described reaction.

15242

A Vaccine Against Japanese B Encephalitis Prepared from Infected Chick Embryos

JOEL WARREN AND RALPH G. HOUGH

(Introduced by J. E. Smadel)

From the Division of Virus and Rickettsial Diseases, Army Medical School, Army Medical Center, Washington, D. C.

Most of the pathogenic viruses affecting man have been cultivated in the developing embryonated egg.¹ In addition the egg has provided a source of vaccine for immunizing human beings against such viruses as yellow fever,² influenza,³ vaccinia,⁴ and equine encephalomyelitis.⁵ Although the virus of Japanese B encephalitis was shown to multiply in the embryonated egg,⁶ growth of this agent in chick embryo tissue was not sufficiently luxuriant to offer promise of providing material for a potent vaccine against this disease. Indeed, the reported experiments re-

garding such a vaccine indicated that it possessed little immunizing capacity.⁷

The vaccines at present employed against Japanese B encephalitis are prepared from infected brain tissue of mice.⁷ These are capable of eliciting neutralizing antibodies against the virus in vaccinated human beings and of inducing resistance to infection in experimental animals. Such vaccines have been prepared commercially and have been authorized for use in the U. S. Army. Although the mouse brain vaccine has desirable immunological properties, it possesses certain potentially objectionable features. For example, it is well known that vaccines containing brain tissue may on occasion produce a demyelinating encephalopathy. In addition the technical difficulties inherent in the large scale manufacture of such a vaccine are considerable. Because of these disadvantages it seemed worth while to reinvestigate the possibilities of obtaining a potent vaccine from tissues of infected chick embryos.

¹ Burnet, F. M., Med. Research Council, Special Report No. 220, 1936, London.

² Theiler, M., and Smith, H. H., *J. Exp. Med.*, 1937, **65**, 787.

³ Burnet, F. M., and Lush, D., *Brit. J. Exp. Path.*, 1938, **19**, 17.

⁴ Goodpasture, E. W., Buddingh, G. J., Richardson, L., and Anderson, K., *Am. J. Hyg.*, 1935, **21**, 319.

⁵ Beard, J. W., Finklestein, H., Sealy, W. C., and Wyckoff, R. W., *Science*, 1938, **87**, 490.

⁶ Hargen, E., and Crodel, B., *Zent. Bakt., Abt. 1*, orig., 1938, **142**, 269.

⁷ Sabin, A. B., Duff, C., Warren, J., Ward, R., Peck, J. L., and Ruchman, I., *J. A. M. A.*, 1943, **122**, 477.



Fig 1

Rabbit eye 4 days after injection of the virus of eastern equine encephalomyelitis into the anterior chamber (K513)

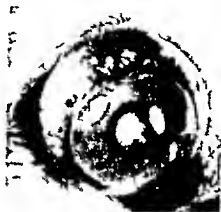


Fig 2

Rabbit eye showing minimal cloudiness of cornea with a typical hyperemia of subconjunctival vessels associated with uveitis K424, 3 days after injection of western equine encephalomyelitis virus

characteristic corneal opacity, usually of severe degree. The inoculum in each case was a 10% suspension of infected mouse brain. Titrations of the inocula by intracerebral injection in mice have shown that a 10^{-7} dilution was regularly fatal and a 10^{-8} dilution occasionally killed mice.

Tests for virus in the aqueous humor and/or ocular tissues have been made at 2, 4 and 11 days. Results as presented in Table II show that a negligible amount of virus remained. This was true even in an

animal suffering from a severe encephalitic infection.

Of the 5 rabbits injected with the eastern virus, 2 were killed at 2 and 4 days and 1 of the remaining 3 developed encephalitis.

Discussion From the data presented in Tables I and II, it is clear that the corneal opacity observed in rabbits after intraocular injection of the viruses of western equine encephalomyelitis is not the result of the growth of virus in the eye, even though such growth may occur to a limited extent. In control experiments the injection of suspensions of normal brain and of numerous other non-viral preparations into rabbits' eyes has never in our experience caused the type of ocular reaction that characterized the experiments with these viruses or influenza viruses. As in the experiments with influenza virus, large doses of equine virus (in the vicinity of a million mld for mice) must be injected in order to produce the corneal reaction. In most of our experiments, the dosage of western virus was apparently close to the minimum level necessary to produce a reaction inasmuch as frequently some eyes were negative while others were positive. The minimum dose of eastern virus that will cause the reaction was not determined.

In the present paper, attention is centered on the corneal reaction because this is the most prominent and most characteristic change observed. A uveitis is less specific but was even more regularly present as indicated by the occurrence of hyperemia and deep radial folds in the iris and a corona of dilated vessels visible around the cornea and extending back over the bulbar con-

TABLE II
Tests for Eastern Equine Encephalomyelitis in Rabbit Eyes After Injection of 0.2 ml of 10% Infected Mouse Brain

Rabbit No	Eye	Material tested	Interval, days	Fate of mice injected with dilutions			Cornea
				10-0	10-1	10-2	
K510	Rt	Aq h	2	2 2 3	3 4 s	s s s	Opaque
	"	Tissue	"	—	2 2 6	s s s	"
	Left	"	"	2 s s	s s s	s s s	"
K511	Rt	Aq h	4	s s s	s s s	s s s	"
	"	Tissue	"	—	4 s s	s s s	"
	L	Aq h	"	s s s	s s s	s s s	"
K531*	Rt	"	11	s s s	—	s s s	"
	L	"	"	s s s	—	s s s	Opaque at 3 days Cleared

* Severe encephalitis

TABLE I
Japanese Encephalitis
The Immunizing Capacity of Vaccines Prepared from the Chick Embryo

Egg passage No	Type of vaccine	Incubation temp., °C	Infectivity titer before inactivation	Minimal immunogenic dose, 50% end point cc
14	10% embryo	37.5	10 ^{-7.0}	0.57
17	" "	37.5	10 ^{-7.4}	0.043
22	" "	35.0	10 ^{-7.5}	0.038
24	" "	35.0	10 ^{-7.3}	0.060
28	" "	37.5	10 ^{-7.0}	0.057
31	" "	37.5	10 ^{-7.7}	0.038
32B 1	20% "	35.0	10 ^{-8.4}	0.006
32B 2	" "	37.5	10 ^{-7.7}	0.012
39A 1	" "	35.0	10 ^{-7.5}	0.009
39A 2	" "	37.5	10 ^{-7.5}	0.016
39	" "	35.0	10 ^{-8.2}	0.008
43	" "	35.0	10 ^{-7.7}	0.0048
43A	" "	35.0	10 ^{-7.8}	0.0043
Pool 50 52	" "	35.0	10 ^{-7.7}	0.121
50B	" "	35.0	10 ^{-7.4}	0.0023
51	" "	35.0	10 ^{-8.0}	0.0100
51A	" "	35.0	10 ^{-7.7}	0.008

of infected mouse brain simultaneously tested averaged 1.1 mg/cc.) The method employed in the assays of the Japanese B encephalitis vaccines was a simplified procedure used by A. B. Sabin⁹ in routine laboratory tests on experimental vaccines. It differs from the standard method he developed for routine assay of mouse brain vaccines for commercial purposes in that fewer mice are used and the challenge dose of virus is not titrated intraperitoneally. However when assays on chick embryo vaccines were performed at the National Institute of Health using the standard method, the results agreed closely with tests on the same material done at the Army Medical School.

The method we have employed is as follows. Graded amounts of vaccine are inoculated intraperitoneally into groups of 10 mice weighing 18 to 20 g each. The dose is repeated 3 days later. One week after the first dose of vaccine, the mice are injected intraperitoneally with a challenge inoculation consisting of 0.3 cc of a 10% suspension of infected mouse brain. In order for a test to be considered valid the challenge inoculum must have an intracerebral titer of 10^{-8.0} or more and must kill at least 80% of 20 non-vaccinated mice which are included in each test

as a control. Test mice are observed for a period of 3 weeks and the deaths are recorded. The 50% end-point method of calculation is applied to the data to determine the amount of vaccine required to protect half of the mice against the challenge inoculation. This quantity is designated as the Minimal Immunogenic Dose.

The present standard of potency for Japanese B encephalitis vaccines which has been adopted by the National Institute of Health and by the Office of the Surgeon General, U. S. Army,¹⁰ requires that a total inoculum of not more than 0.01 cc of vaccine protect at least 50% of the vaccinated mice.

Results. Vaccines against Japanese B encephalitis have been prepared from chick embryo which meet the standards acceptable for mouse brain vaccine, see Table I.

It will be noted that the vaccines which meet these requirements all contained 20% infected embryo tissue. Thus, 8 vaccines of a group of 11 which contained 20% tissue assayed 0.01 cc or less, while all 6 of the 10% vaccines were unacceptable. The 10% vaccines were prepared from eggs infected with early passage material, and the infectious

¹⁰ Federal Security Agency, National Institute of Health, *Minimum Requirements Japanese B Encephalitis Vaccine*, Revision of 5 February, 1945.

⁹ Sabin, A. B., personal communication.

The present report describes the development and preparation of such a vaccine which, in its capacity to immunize mice, compares favorably with the commercial Japanese B vaccines currently made from infected mouse tissue

Cultivation of the Virus The "Nakayama" strain of virus was established and carried in embryonated eggs by the inoculation of 0.1 cc amounts of infected allantoic fluid or embryo suspension into the chorio-allantoic sac. Death of the embryo occurred in practically all the inoculated eggs between the fourth and the sixth day.

Several experiments were performed to determine the optimal conditions for growth of Japanese B encephalitis virus in the developing egg. The results may be summarized briefly as follows. The amount of virus in the inoculum and the route by which it was injected had no marked effect upon the yield of virus, provided the agent was inoculated beyond the chorio-allantoic membranes. The agent was present in all tissues and fluids of the inoculated embryo, but the embryo regularly yielded more virus than the fluids or membranes. The infectious titers obtained in one experiment illustrate this point; the results were, embryo $10^{-7.0}$, yolk sac $10^{-5.0}$, allantoic fluid $10^{-4.6}$, chorio-allantoic membranes $10^{-4.3}$, and yolk fluid $10^{-4.0}$. The maximum concentration of virus in the embryo or in the allantoic fluid of inoculated eggs was obtained after incubation at 35°C for 48 to 72 hours. It is of interest to note that during the course of serial passage of the virus in eggs, the infectivity of inoculated embryo increased. By the thirtieth passage, the infective titers of embryo suspensions ranged between $10^{-7.5}$ and $10^{-8.5}$, with an average value of about $10^{-7.8}$.

Preparation and Assay of Vaccines The seed virus to be used for inoculation of eggs was harvested and stored as a 10% suspension of infected embryo tissue in whole filtered chicken serum. Such seed suspensions withstood storage at -70°C in sealed ampoules for more than 1 month with no appreciable loss in titer. Both seed virus and

material for vaccine were obtained from embryonated eggs which were 7 or 8 days old when infected via the chorio-allantoic sac. These were subsequently incubated at 35°C for 3 days and at the end of this time the embryos were harvested.

The present method for preparing formalinized vaccine from the infected embryo is now given in detail. The whole embryo was removed from groups of 20 to 200 infected eggs. The lens and retinal cup were dissected out and discarded. The remaining embryonic tissue was then pooled and homogenized in a Waring blender for 5 minutes with sufficient physiological saline, buffered at pH 7.4, to make a 10 or 20% suspension by weight. The resulting suspension was strained through gauze. After samples were removed for sterility tests and for titration of infectivity, sufficient formaldehyde U.S.P. was added to give a final concentration of 0.2%.*

The material was then stored at 5°C for 2 weeks. It was then assayed for potency and a safety test was performed. For the latter test, 10 mice were inoculated intracerebrally with the vaccine and observed for signs of neurological disease for a period of 2 weeks in order to determine that inactivation of the virus had been complete. The protein nitrogen content of seven samples of 20% embryo vaccines was determined; the values ranged between 0.68 mg/cc and 0.95 mg/cc with an average of 0.84 mg/cc. (The protein nitrogen in a 10% suspension

Experiments were performed to determine the comparative effectiveness of formaldehyde and ultraviolet light as inactivating agents on allantoic fluid vaccines. The minimal lethal concentration or exposure time was first determined for each agent. The ultraviolet source was a water-cooled mercury vapor lamp of the type described by Oppenheimer and Levinson.⁸ Under the conditions of these experiments formaldehyde treated vaccine yielded superior antigenic potency. The inactivation experiments were performed in collaboration with Dr. Fred Stumpert of the Puoke, Davis Co., Detroit, Mich.

⁸ Oppenheimer, F., and Levinson, S. O., cited in *J. Immunol.*, 1945, 50, 317 (original paper unpublished).

now in progress to determine whether other agents will serve as satisfactory adjuvants with Japanese encephalitis vaccine

At the present time no information is available regarding the capacity of this vaccine either alone or with adjuvants to immunize man

Summary 1 Highly infectious suspensions of embryo tissue have been prepared from embryonated chick eggs inoculated with

the Japanese B virus Infective titers of $10^{-7.5}$ to $10^{-8.5}$ were regularly encountered under suitable conditions

2 Formalinized vaccines made from 20% suspensions of infected chick embryo tissue are capable of immunizing mice against intraperitoneal infection with the virus of Japanese B encephalitis Such vaccines are comparable in potency to the Japanese B encephalitis vaccines prepared from infected mouse brain and used by the U S Army

15243 P

Cytotoxic Property of Mouse Cancer Antiserum *

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In our investigations of the milk factor,¹ or the Bittner virus, associated with mammary carcinoma of mice, an antiserum prepared from rabbits has been tested for its toxicity for cancer cells In his studies, Andervont² found that an antiserum prepared in rabbits by injecting as antigens the centrifugates of mouse tumor, neutralized the milk factor, while normal serums failed to do so Green, Moosey and Bittner³ likewise have shown that antisera prepared from immunized rabbits, as well as from immunized rats, neutralized the agent We have prepared additional control serums by immunizing rats and rabbits against normal mouse tissue centrifugates, and these have failed to neutralize the virus This finding demonstrates that the milk factor is antigenically unlike mouse tissue and might more properly be considered a foreign virus In

the experiments here reported a rabbit antiserum prepared against the Bittner virus has been tested for its toxic properties for mammary cancer cells when mixed with them *in vitro*

Three rabbits were immunized by 5 weekly injections of cancer centrifugate given intraperitoneally A suspension of mouse cancer tissue was homogenized and subjected to fractional centrifugation The first sediment, obtained by centrifugation for 1½ hours at 14,000 X gravity, was discarded The supernatant suspension was again centrifuged 1 hour at 100,000 X gravity This second sediment was collected, suspended in a 0.9% saline solution and injected in a dosage corresponding to 2 g of original tumor tissue The rabbits were bled for serum 14 days after the fifth injection of centrifugate An antiserum against normal lactating mouse mammary tissue was prepared in an identical manner by subjecting lactating breast tissue to the same processes The normal breast tissue was obtained from mice susceptible to, but not carrying, the Bittner virus An additional control serum was made from normal rabbit blood Each of the 3 serums, as finally prepared, represented a pooled serum from 3 rabbits The 3 serums were test-

* Aided by grants from the Cancer Research Fund, Graduate School of the University of Minnesota, and the Jane Coffin Childs Memorial Fund for Medical Research

¹ Bittner, J J, *Science*, 1936, **84**, 162

² Andervont, H B, and Bryan, W R, *J Nat Cancer Inst*, 1944, **5**, 143

³ Green, R G, Moosey, M M, and Bittner, J J, *Cancer Research*, 1945, **5**, 588

TABLE II
Effect of an Adjuvant ("Falba" and Mineral Oil) on the Immunizing Capacity of Certain Japanese B Vaccines

Vaccine tested	M I D vaccine assayed in	
	Saline, cc	2% "Falba" Min oil cc
No 22—10% chick embryo	0 038	0 017
No 32B—20% chick embryo	0 006	0 0025
Pool II—20% chick embryo—crude	0 011	0 0029
Pool II—20% chick embryo—angle centrifuged	0 013	0 004
No 43—10% mouse brain	0 040	<0 0012

titers of the tissues used in their manufacture were generally somewhat lower than the titers of tissues which were used for the 20% preparations. That there was some correlation between the infectivity of the embryo tissue and the potency of the vaccine prepared from it, is clearly demonstrated in Table I. Thus, all 5 of the 20% vaccines made from tissues with infective titers greater than $10^{-7.5}$ were acceptable when assayed while only three of the 6 similar preparations with infective titers before inactivation of $10^{-7.3}$ to $10^{-7.5}$ were satisfactory. Further evidence for the border line immunogenic properties of vaccines made from tissues with titers of $10^{-7.0}$ to $10^{-7.5}$ was found in the unsatisfactory results obtained with the 10% vaccines. Finally, several vaccines prepared from chorio-allantoic fluids with titers of $10^{-6.5}$ or less were unacceptable when assayed.

By means of adsorption, centrifugation, and the use of adjuvants, attempts were made to increase the immunizing capacity of the embryo vaccine. Concentration of the antigenic material of the vaccines could not be obtained with adsorption on calcium phosphate or potassium alum. High speed centrifugation in the Sharples† or the angle ultra-centrifuge removed most of the antigen from the supernatant fluid of both allantoic fluid and 10% embryo vaccines but the sediment did not exhibit the expected increase in potency.

For example, the sediment obtained on high speed centrifugation of a 10% embryo

vaccine was resuspended in sufficient saline solution to make 1/10th the original volume, this had a two- to three-fold increase in immunizing capacity over the original vaccine and contained about one and a half times the amount of protein nitrogen of that in the starting material. Thus, the immunizing agent had been concentrated in the ultra-sediment and perhaps somewhat purified.

A mixture of "Falba"‡ and mineral oil has been used as an adjuvant to augment the immunizing capacity of certain bacterial and protozoal antigens¹¹ and of influenza vaccines for human beings¹². We have obtained encouraging results with this adjuvant employed in conjunction with Japanese B embryo vaccines, see Table II.

A mixture of equal parts of "Falba" and mineral oil was prepared and a 2% emulsion of this material in physiological saline solution was used as a diluent for the vaccines throughout the assay procedure. Control assays were done on the same lots in the ordinary manner, *i.e.* dilutions were made in saline solution. It is evident from the results presented in Table II that the minimal immunogenic dose of a given vaccine was appreciably less when the adjuvant was employed. It may be pointed out that vaccines containing "Falba" and mineral oil elicit certain untoward reactions when injected into human beings¹². For this reason studies are

† The Sharples centrifugation experiments were performed with the kind assistance of Dr W M Stanley, Rockefeller Institute for Medical Research, Princeton, N J

‡ "Falba" is the proprietary name for a compound prepared from lanolin and composed of a mixture of "oxycholestrins and cholestrins."

¹¹ Freund, J, and McDermott, K, *Proc Soc Exp Biol and Med*, 1942, **49**, 548

¹² Henle, W, and Henle, G, *Proc Soc Exp Biol and Med*, 1945, **59**, 179

Antigenic Character of the Cancer Milk Agent in Mice *

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The influence of milk in the transmission of mammary cancer in mice described by Bittner¹ was determined by him to be filterable.^{2,3} The colloidal dimension of the influence was ascertained by means of ultracentrifugation by Visscher, Green, Bittner, Ball and Siedentopf,⁴ and these authors suggested that the agent might be classed as a filterable virus. Additional data showing that the agent is filterable have been presented by Andervont and Bryan⁵ and by Bittner, Evans and Green.⁶ We have pursued studies on the antigenic character of the milk agent to gain further information as to its general characterization and in particular as to its properties as a virus.

Studies on the antigenic nature of the mouse-tumor agent have been pursued by Andervont and Bryan,⁵ who have recently reported on the neutralization of the milk influence by immune serums. Two immune serums were prepared by them, each from one rabbit. These serums apparently neutralized the mouse mammary-tumor milk agent, as only 1 of 36 mice injected with tumor material mixed with the antisera developed a tumor, while 10 of 15 mice injected with tumor material mixed with nor-

mal rabbit serum developed tumors.

In our investigations of the antigenic character of the milk agent, we have utilized as controls the agent suspended in saline solution and the agent treated with normal serum and with serum from animals immunized with normal tissue. As the agent we have used high-speed centrifugates collected between a first run at 15,000 g and a second at 95,000 g. These centrifugates which were previously demonstrated⁴ to contain much of the milk agent, have been injected into rabbits and white rats to produce antisera. Five doses of centrifugate, each consisting of a 4-g equivalent of spontaneous mouse tumor suspended in 3.5 cc of saline solution, were injected into rabbits at 5-day intervals. The rats were immunized similarly, except that each immunizing dose was 2 cc and contained the equivalent of 1 g of mammary-tumor tissue. The animals were bled for the production of antiserum from 7 to 10 days after the last injection. In the first experiment on the neutralization of the agent, 90 mice were injected in 3 groups of 30 mice each. Group 1 was injected with a centrifugate equivalent to 0.25 g of tumor tissue suspended in 0.5 cc saline solution. At the end of 11½ months, 25 of the 30 mice had developed mammary cancer. The 30 mice in Group 2 were injected with a centrifugate equivalent to 0.25 g of tumor tissue suspended in 0.5 cc of rabbit immune serum after the mixture had stood at room temperature 2 hours. The antiserum was a pooled mixture of the serums of 3 rabbits immunized as described above. At 11½ months, 2 of the 30 mice had developed tumors. The 30 mice in Group 3 were injected with similar amounts of centrifugate suspended in 0.5 cc of normal rabbit serum. Of these, 14 had developed breast cancer at 11½ months. All inoculated mice in these groups were of the

* This investigation has been aided by grants from the Jane Coffin Childs Memorial Fund for Medical Research, the Cancer Research Fund of the Graduate School of the University of Minnesota, and the Citizens Aid Society of Minneapolis.

¹ Bittner, J. J., *Science*, 1936, **84**, 162.

² Bittner, J. J., *Science*, 1942, **95**, 462.

³ Bittner, J. J., *Cancer Research*, 1942, **2**, 710.

⁴ Visscher, M. B., Green, R. G., Bittner, J. J., Ball, Z. B., and Siedentopf, H. A. *Proc. Soc. Exp. Biol. and Med.*, 1942, **49**, 94.

⁵ Andervont, H. B., and Bryan, W. R. *J. Nat. Cancer Inst.*, 1944, **3**, 143.

⁶ Bittner, J. J., Evans, C. A., and Green, R. G., *Science*, 1945, **101**, 95.

TABLE I
Cytotoxic Property of Mouse Cancer Antiserum
Rabbit antiserum—C3H tumor—ZBC mice without milk factor

No mice	Tumor cell suspension injected with	No tumors in			
		10	15	40	60 days
10	Cancer antiserum	0	0	0	0
8*	Normal mammary antiserum	1	3	8	8
10	Normal rabbit serum	1	1	5	7
10	Saline sol	10	10	10	10

* Two died from intercurrent infection

ed for their comparative cytotoxicity

To test the serums, a cancer-cell suspension for the production of transplant tumors was obtained as follows. A mass of cancer tissue was forced through a tissue press. The macerated cancer tissue was then suspended in saline solution and allowed to settle for 30 minutes. A heavy sediment of tissue masses settled out and the supernatant suspension of cancer cells and small cellular masses was removed for injection into susceptible mice. The concentrated cancer-cell suspension was then diluted 10 times. To 4 portions of the cell suspension were added, respectively, 5 volumes of cancer antiserum, normal tissue antiserum, normal rabbit serum, and 0.9% saline solution. All mixtures were treated in the same manner. They were held at room temperature 3 hours, and then in a refrigerator at 7° C for another 3-hour period. Each preparation was then injected in a dosage of 0.6 cc, intraperitoneally, into a separate group of 10 mice. The dose given each mouse represented 0.1 cc of a 1 to 10 suspension of cancer cells and 0.5 cc serum or 0.9% saline solution.

The formation of tumors at various intervals is shown in Table I.

It is evident from the data that the cancer antiserum completely inhibited growth of cancer cells. Both the normal rabbit serum and the normal mammary tissue antiserum ef-

ected a delay in the development of tumors. The normal rabbit serum appears to have inhibited slightly the growth of tumor cells, since only 7 mice of the 10 injected displayed tumors at the end of 60 days.

Tumors developed in all mice injected with tumor cells in saline solution much more rapidly than they developed in mice receiving cancer cells in normal rabbit serum or in normal mammary tissue antiserum. There appears, therefore, to be something in normal rabbit serum which interferes with the rapid growth of cancer cells. This effect of normal serum is not increased, but possibly decreased, by immunization with normal tissue centrifugates.

These results seem to demonstrate that an antiserum produced from mouse cancer-cell centrifugates is capable not only of neutralizing the virus but also of inhibiting the growth of cancer cells produced by the virus.

Conclusions An immune serum prepared in rabbits against the mouse mammary carcinoma milk factor, or Bittner virus, when mixed with cancer cells for a period of 6 hours completely inhibits their growth so that transplant tumors do not develop. Control tumors developed after similar treatment of tumor cells with serum from normal rabbits and with serum from rabbits immunized with normal lactating mouse breast tissue.

serums effected a retarding of tumor development to a rate below that observed with the agent in saline solution, and ultimately a smaller number of tumors was produced

Conclusions The milk factor of mammary mouse carcinoma is highly antigenic and stimulates the formation of antibodies in both rabbits and rats. The mouse cancer antiserum neutralizes and renders inactive

mouse cancer centrifugates. Equally effective antisera are prepared from spontaneous and from transplant tumors. Normal rabbit and rat serums have a slight effect in inactivating the mouse cancer virus. The finding that antiserum against normal mouse tissue does not have any neutralizing or adverse effect on the milk agent indicates that the agent is a virus of exogenous origin.

15245

Cell-Blockade in Canine Distemper *

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The interference phenomenon of animal viruses was first observed by Hoskins¹ and by Magrassi² independently. Magrassi observed an interference between nonencephalitic and encephalitic herpes viruses in rabbits, and Hoskins reported an interference between neurotropic and pantropic yellow fever viruses in monkeys. Subsequent investigations by Findlay and MacCallum,³ Dalldorf,⁴ Jungeblut and Sanders,⁵ Ziegler, Lavin and Horsfall,⁶ the Henles,⁷ and others have shown that the cell-blockade, or interference phenomenon, can occur in many animal viruses under experimental conditions.

We have conducted experimental studies

* Aided by a grant from the Medical Research Fund of the Graduate School of the University of Minnesota and by experimental facilities and financial support furnished by Fromm Laboratories, Inc., Grifton, Wise.

¹ Hoskins, M., *Am J Trop Med*, 1935, **15**, 675.

² Magrassi, F., *Z f Hyg u Infektionskr*, 1935, **117**, 501, 573.

³ Findlay, G. M., and MacCallum, F. O., *J Pathol and Bact*, 1937, **44**, 405.

⁴ Dalldorf, G., *J Imm*, 1939, **37**, 245.

⁵ Jungeblut, C. W., and Sanders, M., *J Exp Med*, 1942, **76**, 127.

⁶ Ziegler, J. L., Jr., Lavin, G. I., and Horsfall, F. L., *J Exp Med*, 1944, **79**, 379.

⁷ Henle, W., and Henle, G., *Am J Med Sci*, 1944, **207**, 705.

of simultaneous infections of young foxes with virulent distemper virus and a distemper virus modified by ferret passage, and found that the phenomenon of interference, or cell-blockade, can be demonstrated for these viruses. Foxes inoculated *intranasally* with virulent distemper virus can be protected from fatal infection by subsequent inoculation of the ferret-passage distemper virus, which is of low virulence for foxes and dogs. In foxes injected *intramuscularly* with the virulent distemper virus, the course of the infection is also blocked by the introduction of the modified virus either 3 days before, or at the same time that, the virulent distemper virus is given. However, if the interval elapsing between the intramuscular injection of the virulent virus and the subsequent inoculation of distemperoid virus is as long as 3 days, there is very little interference with the course of the virulent infection.

Materials and Methods The animals inoculated were red fox pups 10 to 16 weeks old. In any one experiment the foxes were very nearly the same age. The virulent distemper virus was of dog origin, and had been passed experimentally through foxes until it acquired an extremely high virulence for that animal. The ferret-passage virus was the distemperoid virus described by Green.⁸

⁸ Green, R. G., *Am J Hyg*, 1945, **41**, 7.

TABLE I
Neutralization of Mouse Milk Agent with Rat Immune Serum

No mice	Tumor centrifugate mixed with	Tumors produced in mo					
		7½	8	9	10	11½	13½
24	Spontaneous tumor antiserum	0	0	0	0	0	0
24	Transplant tumor antiserum	0	0	0	0	0	0
24	Normal tissue antiserum	1	3	7	10	12	21
24	Normal rat serum	2	4	8	9	12	13
24	0.9% saline sol	6	10	15	15	17	18

Dosage Centrifugate equivalent of 0.2 g tumor tissue mixed with 0.18 cc antiserum in 0.9% saline to make total volume of 0.23 cc. The mixtures were held at room temperature for 2 hours before injection.

Note All injected mice subjected to forced breeding. Mice 1 mo old ZBC stock without milk influence.

ZBC strain, mice which were susceptible to, but did not carry, the tumor agent. After inoculation, the mice were maintained as forced breeders in order to stimulate maximum cancer development. These results confirm the findings of Andervont and Bryan.⁵

A second experiment on the antigenic properties of the milk agent has yielded similar results 14 months after its inception. The antiserum used to neutralize the milk agent in the latter experiment was prepared from laboratory rats as described above. Three antisera were prepared: one from spontaneous tumor tissue, one from transplanted tumor tissue, and one from normal mouse tissue consisting of heart, liver, spleen and kidney. Each antiserum, as finally prepared, was a pooled serum from 3 immunized rats. In addition, a pool of normal rat serum was prepared by combining the sera from 3 un.injected rats. ZBC mice not carrying the milk influence were injected at 1 month of age in groups of 24 with centrifugates of tumor tissue mixed and incubated for 2 hours with the various antisera. One control group received infective centrifugate suspended in 0.9% saline solution. The appearance of tumors at different intervals and the number ultimately produced are shown in Table I.

Of the 2 groups of mice totaling 48 that received spontaneous tumor antiserum and transplanted tumor antiserum, none has developed cancer at 13½ months. Twenty-one mice of the 24 receiving normal tissue antiserum have developed cancer, and 13 of the 24 that received the normal rat serum have developed cancer. Of the 24 control mice,

which received the infective centrifugate without the addition of serum, 18 have become cancerous. These additional data obtained with antisera made from the closely related rat would seem to establish rather definitely that the infective agent in mouse mammary carcinoma is antigenic.

Demonstration that the milk influence of Bittner is an antigen becomes of greatest importance in its characterization. First termed an *influence* and later an *agent* or an *incitor*, its antigenic capacity, together with other attributes, would seem to put it in the class of filterable viruses. Although it is difficult to describe a virus accurately, its most definitive characters are (1) reproduction only in association with living host cells, (2) small size, usually ultramicroscopic, and (3) antigenic capacity. All 3 basic criteria are now known to be met by the milk agent. Inasmuch as many tumors in animals are known to be virus infections, it would seem reasonable from the evidence at hand to consider the mammary cancer of mice an infectious process and its cause a filterable virus.

The antiserum produced in rats by the injection of normal mouse tissues failed to produce any adverse effect upon cancer formation by the agent. Of the 3 groups of controls, those receiving the normal tissue antiserum have presented the largest number of cancerous mice. This indicates that the agent is antigenically different from mouse tissue and is not derived therefrom.

Normal rabbit serum and normal rat serum both seem to contain something that is antagonistic to the cancer agent. Both normal

TABLE II
Results of Intramuscular Inoculations of Foxes with Virulent Distemper Virus and Distemperoid Virus (Exp 2)

Days after inj of viru lent distem per virus	Group 1 b 10 foxes inj with viru lent distemper virus (Controls)		Group 2 b 10 foxes inj with dis temperoid virus 3 days before virulent dis temper virus		Group 3 b 10 foxes inj simultane ously with viru lent distemper virus and dis temperoid virus		Group 4 b 10 foxes inj with dis temperoid virus 3 days after virulent dis temper virus	
	Sick	Deaths	Sick	Deaths	Sick	Deaths	Sick	Deaths
12			Normal					
13	1/10						1/10	
14	3/9	1						1
15	5/9							2
16	5/9							3
17	all	1						2
18		1			2/10			2
19					2/10			
20		1			2/10			
21		2			1/10	1		
22		1			0			
23		1			0			
24		1			0			
25					0			
26					0			
27					0			
28					0			
29					0			
30		1			0			

disease can be blocked off if distemperoid virus is injected during the incubation period or in the early stage of the generalized infection

Interference following intramuscular injection of virulent virus In our second experiment 40 young foxes, in groups of 10, were injected intramuscularly with virulent distemper virus. All 4 groups received the virulent distemper virus at the same time, and 3 of the groups were subsequently given intramuscular injections of distemperoid virus at varying intervals

Group 1-b was injected with virulent distemper virus only. On the 13th day after the inoculations, the animals began to show symptoms. By the 17th day all the animals were sick, and at the end of 30 days all had died of typical distemper. Group 2-b received distemperoid virus 3 days before the inoculation of virulent distemper virus. These animals remained well, showing no symptoms whatsoever of distemper. Group 3-b was inoculated with virulent distemper virus and distemperoid virus simultaneously. Two animals exhibited symptoms on the 18th day

after inoculation. One of them died on the 21st day, while the other recovered. The rest of the foxes in this group showed no symptoms of distemper. Group 4-b received distemperoid virus 3 days after the virulent distemper virus. All the animals showed symptoms by the 14th day, and all died of typical distemper by the 18th day. Details of the occurrence of symptoms, deaths, and recoveries are shown in Table II.

In the third experiment 40 young foxes were also used in groups of 10. The control group received only virulent distemper virus. The other 3 groups were inoculated intramuscularly, first with virulent distemper virus and then with distemperoid virus, which was given to the respective groups 3, 8, and 12 days later.

Group 1-c, the controls, began to show symptoms on the 14th day after the injection of virulent virus. All the animals died of distemper by the 29th day. Group 2-c, which received the distemperoid virus 3 days after the virulent virus, began to exhibit symptoms of distemper on the 12th day. By the 36th day 8 animals had died and 2 had re-

TABLE I

Interference in Foxes between Virulent Distemper Virus Inoculated intranasally and Distemperoid Virus Inoculated Intramuscularly
(Exp 1)

Days after injection of distemper virus	Group 1 a 10 foxes inj with virulent distemper virus (Controls) Sick Deaths		Group 2 a 10 foxes inj simultaneously with virulent distemper virus and distemperoid virus† Sick	Group 3 a 10 foxes inj with distemperoid virus 3 days after virulent distemper virus‡ Sick	Group 4 a 9 foxes† inj with distemperoid virus 12 days after virulent distemper virus‡ Sick
16	1/10*		2/10		
17	1/10		2/10		
18	1/10		2/10		
19	2/10		2/10		1/9
20	3/10		2/10		1/9
21	4/10		3/10		2/9
22	4/10		3/10		2/9
23	6/10		5/10	2/10	4/9
24	6/10		5/10	2/10	4/9
25	6/9	1	5/10	2/10	4/9
26	6/9		5/10	2/10	4/9
27	6/9		2/10	2/10	3/9
28	6/8	1	2/10	2/10	3/9
29	7/7	1	2/10	2/10	3/9
30	6/6	1	2/10	2/10	3/9
31	2/2	4	0/10	0/10	1/9
32	2/2				0/9
33	2/2				
34	2/2				
35	1/1	1			
36		1			

* The denominator indicates the number of foxes living, and the numerator the number sick on any given day

† One fox in this group had a broken leg and was killed

‡ No deaths

The virulent virus was injected in a dosage of 100 mg and the distemperoid virus in a dosage of 200 mg. All animals were observed daily for symptoms of distemper. When deaths occurred, the disease was identified by the presence of cytoplasmic inclusions in the epithelial cells of the bladder or the trachea.

Interference following intranasal inoculation with virulent virus. Forty red fox pups were divided into groups of 10. Group 1-a, which received virulent distemper virus only, was inoculated intranasally. Animals in this group exhibited symptoms of distemper by the 16th day, and all died of distemper between the 25th and 36th days. The other 3 groups received the same virus intranasally but were given distemperoid virus in addition. Group 2-a received simultaneous inoculations of the two viruses. Two foxes showed symptoms on the 16th day, and by the 23rd day

5 showed mild signs of the disease. All animals had recovered by the 31st day. Group 3-a was given the distemperoid virus 3 days after injection of the virulent virus. Only 2 animals in this group displayed symptoms, which were discernible on the 23rd day. Both were normal by the 31st day. Group 4-a was given distemperoid virus 12 days after receiving the virulent virus. Mild symptoms of distemper were shown by one animal in this group on the 19th day and by 4 animals on the 23rd day. All had recovered by the 32nd day. Details regarding the occurrence of symptoms, deaths, and recoveries are shown in Table I.

These results demonstrate a very decisive interference, or blocking effect, of distemperoid virus on the course of a virulent distemper infection. They show also that a virulent infection corresponding to the natural

TABLE IV
Comparison of Intramuscular and Intranasal Inoculations of Foxes with Virulent Distemper Virus in Interference Experiments*

Inocula	40 foxes inj intramuscularly			40 foxes inj intramuse			40 foxes inj intranasally		
	No injected	No sick	No dead	No injected	No sick	No dead	No injected	No sick	No dead
Virulent distemper virus only	10	10	10	10	10	10	10	10	10
Distemperoid virus 3 days before dis temper virus	10	0	0						
Distemperoid virus and distemper virus simultaneously	10	2	1				10	5	0
Distemperoid virus 3 days after dis temper virus	10	10	10	10	10	8	10	2	0
Distemperoid virus 8 days after dis temper virus				10	10	9			
Distemperoid virus 12 days after dis temper virus				9†	9	9	9†	4	0

* All distemperoid virus injected intramuscularly

† One fox had a broken leg and was killed

The results obtained in the case of *intramuscular* inoculation of the virulent virus are similar, provided the modified virus is given before, or even at the same time as, the virulent virus. A striking difference, however, is observed in the number of recoveries of treated foxes when the modified virus is given after intramuscular injection of virulent virus. In this case, little or no interference follows the injection of the modified virus.

It appears that protection occurred when the modified virus was given an opportunity to reach tissue cells ahead of the virulent virus. On the other hand, if the virulent virus was able to invade a majority of susceptible cells before the modified virus, no protection occurred. In the intramuscular inoculations the virulent virus, when given first, seems to have immediately entered the blood stream as free virus and to have been distributed rapidly to large numbers of tissue cells. However, in the intranasal inoculations, the virulent virus appears to have invaded the animal body slowly, perhaps principally through neighboring tissue cells. At least, it did not seem to have become free virus in the blood stream and to have invaded large numbers of tissue cells ahead of the later-injected modified virus. The mechanism appears to be a reciprocal cell-block. Either

the modified virus will be blocked by the virulent virus, if the virulent virus has already invaded the susceptible cells, or the modified virus, if it gets to the tissue cells first, will block the effect of the virulent virus on those cells. All our experimental results point to a mechanism of cell-blockade rather than to interference by one virus directly with the other.

Summary A modified ferret-passage distemper virus exhibits an interference, or cell-blockade, phenomenon with respect to a virulent distemper virus in foxes. If the virulent virus is inoculated intranasally, as occurs in a natural infection, interference occurs when the modified virus is inoculated at the same time as, or after, the virulent virus. If the virulent virus is inoculated intramuscularly, the virulent infection can be blocked off by the modified virus only if the modified virus is given before, or at the same time as, the virulent virus. After an intramuscular injection of a virulent virus, any effect of the modified virus is in turn blocked off. The results appear to be determined by the virus that seeds the most tissue cells first. In the case of intranasal inoculation, the distemperoid virus seems to have a definite therapeutic effect during the incubation period and in the stage of early symptoms.

TABLE III
Results of Intramuscular Inoculations of Foxes with Virulent Distemper Virus and Distemperoid Virus (Exp 3)

Days after inj of dis temper virus	Group 1 c 10 foxes inj with viru lent distemper virus (Controls)		Group 2 c 10 foxes inj with dis temperoid virus 3 days after virulent dis temper virus		Group 3 c 10 foxes inj with dis temperoid virus 8 days after virulent dis temper virus		Group 4 c 9 foxes* inj with dis temperoid virus 12 days after virulent dis temper virus	
	Sick	Deaths	Sick	Deaths	Sick	Deaths	Sick	Deaths
12			4/10				2/9	
13			5/10				2/8	1
14	4/10		8/10		4/10		3/7	1
15	6/10		9/10		5/10		3/7	
16	all	2	all	3	5/10		3/7	
17	"		"		6/10		all	2
18	"		"		6/10		"	1
19	"	1	"		5/9	1	"	2
20	"	3	"	2	5/9		"	
21	"	1	"	1	4/7	2	"	
23	"		"		4/6	1	"	1
24	"		"		4/6			1
25	"	1	"		all	1		
26	"	1	"		"			
27	"		"		"	1		
28	"		"		"	1		
29		1	"		"			
30			"		"	1		
31			"	1	"	1		
36			0/2	1	0/1			
Total No deaths		10		8		9		9

* One fox in this group had a broken leg and was killed

covered Group 3-c received the distemperoid virus 8 days after the virulent virus. Symptoms first appeared on the 14th day. Nine animals were dead by the 31st day, and 1 had recovered. Group 4-c, inoculated with the distemperoid virus 12 days after the inoculation of virulent virus, began to show symptoms on the 12th day. All animals in this group were dead by the 24th day. Details of the symptoms, deaths, and recoveries are shown in Table III.

The first experiment on the intramuscular injection of virulent virus demonstrated that the distemperoid virus completely protected foxes when it was inoculated before the virulent distemper virus and gave almost complete protection when the inoculations were simultaneous. Both of the experiments on intramuscular injections included a group inoculated with distemperoid virus 3 days after the virulent virus was given. In both

cases the controls died within approximately the same length of time. Some differences were observed in the 3-day groups. In Experiment 2 all the animals died but in Experiment 3 only 8 of 10 animals died. The recovery of 2 animals appeared due to protection, since foxes seldom recover once they have exhibited symptoms of virulent distemper. In the second intramuscular experiment little or no protection was apparent in the 8-day group and no protection in the 12-day group. A comparison of the results obtained from intranasal and intramuscular inoculations of virulent distemper virus is shown in Table IV.

Discussion Our results seem to show that when the virulent distemper virus is inoculated *intranasally*, an injection of the modified virus given at the same time or 3 to 12 days later, so interferes with, or blocks off, the virulent infection that severe symptoms do not develop and recovery is rapid and complete.

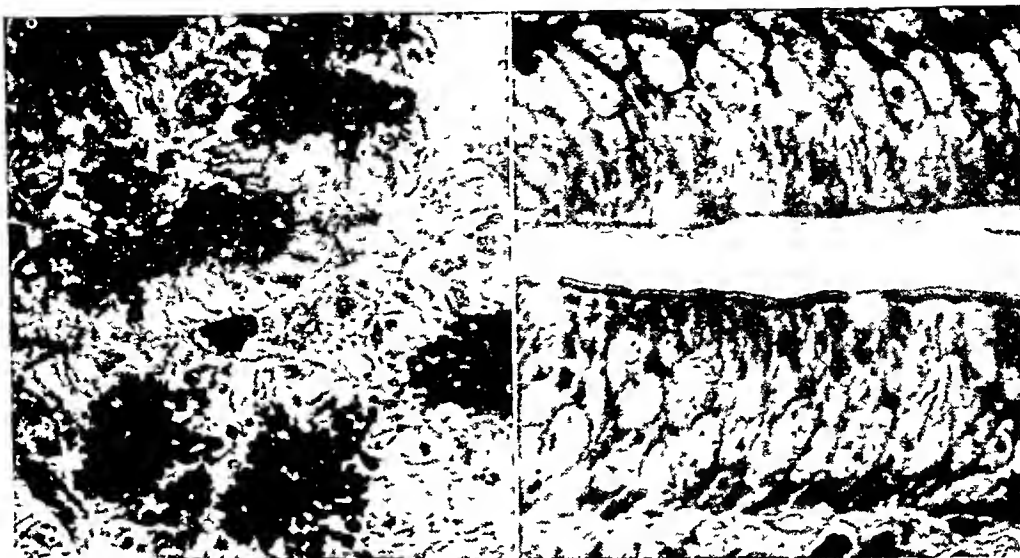


FIG 1

A section of pancreas of a non fasted adult guinea pig showing the centrally located zymogen granules and the more peripherally located elongated mitochondria. Fixed in 9% resorcinol in 4% alkalinized formaldehyde, sectioned at 2 micra and stained by the Altmann-Bensley procedure. Mag. $\times 2100$.

FIG 2

A section of epithelium from the duodenum of a non fasted mouse showing particularly the mitochondria and the brush border of the cells. Fixed in 7% pyrogallol in 4% alkalinized formaldehyde, sectioned at 2 micra and stained by the Altmann-Bensley procedure. Mag. $\times 2100$.

Photomicrographs taken by H. W. Morris

1 to 2 micra without much difficulty. The reaction of various staining procedures following this fixation is interesting. The iron-hematoxylin methods give the best results. Mitochondria and other cytoplasmic granules stain sharply and the nuclei show good detail as do such structures as basement membranes, terminal bars, and brush borders. The Altmann-Bensley staining procedure also gives good results if the tissue slices are not treated with potassium permanganate and oxalic acid or with potassium dichromate. Schleicher's modification of Mallory-Heidenhain connective tissue stain² gives fairly good results although the azocarmine overstains to some extent, but routine hematoxylin and eosin methods must be preceded by treatment with potassium permanganate and oxalic acid to insure staining with the hematoxylin. After

the tissues have been stored in alcohol for some time and have darkened considerably the results obtained with all of the above staining procedures except iron-hematoxylin, are less satisfactory.

Resorcinol-formol Mixtures The procedure for the preparation of resorcinol-formol mixtures is the same as that for the pyrogallol-formol solutions already outlined. Tissue pieces are well fixed in solutions of 4.5 to 9% resorcinol in 4% alkalinized formaldehyde. The time necessary for fixation and the subsequent treatment of the tissue blocks are the same as for the pyrogallol-formol mixtures but tissue fixed with resorcinol-formol darkens much more slowly when stored in alcohol so that storage may be somewhat longer without altering the staining reactions.

Tissue prepared in this manner is somewhat softer and sections with greater ease than when fixed with pyrogallol-formol solu-

² Schleicher, E. M. *Am. J. Clin. Path.* (Tech. Suppl.) 1943, 7, 35.

Hydroxybenzene Compounds as Cytoplasmic Fixatives *

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Because of a renewed interest in cytoplasmic components quickened by the development of methods for centrifugal fractionation of these constituents, this investigation was undertaken in an effort to develop a simple yet effective histological method for the study of the cell cytoplasm and, more particularly, of such structures as the mitochondria. At the present time 3 types of agents¹ are employed in fixation of the cytoplasm, heavy metals, osmium tetroxide, and formaldehyde. As the protein precipitating properties of the heavy metals have been investigated rather extensively, further consideration of these compounds did not appear to be warranted. Osmium tetroxide gives adequate preservation of only a small layer of cells within a tissue block, its "fixing" properties possibly being due to its action as a strong oxidant. The uncontrollable nature of such oxidation, however, severely limits the usefulness of this reaction in the fixation of tissues. Formaldehyde, on the other hand, is a weak reducing agent, and although in itself it does not yield exceptional results, suggests that other reducing compounds might be of value in the preservation of cytoplasmic constituents.

In line with this reasoning, trihydroxybenzene or pyrogallol was considered as it seemed to be well suited for our purposes, being a relatively strong reducing substance, when in alkaline solution, as is required for the maintenance of cytoplasmic detail. Preliminary investigations revealed that weak solutions of pyrogallol preserved the mitochondria but caused excessive alteration of

other cell structures. As hydroxybenzene compounds combine with formaldehyde, pyrogallol-formol mixtures were then tried and found to give more desirable results. Investigation of the unsubstituted dihydroxybenzenes in combination with formaldehyde revealed that hydroquinone and catechol give rather poor fixation but that resorcinol approximates pyrogallol in effectiveness.

Pyrogallol-formol Mixtures As alkaline solutions of pyrogallol and formaldehyde take up oxygen readily and acid solutions form high polymers on standing, all solutions must be made up shortly before use. This is a relatively minor disadvantage, however, since weighed amounts of pyrogallol can be stored in the dry vials in which the tissue is to be fixed, and the desired amount of alkalized formaldehyde solution added just prior to use.

For the fixation of tissue pieces, up to 5mm in thickness, solutions of 3.5 to 7% pyrogallol dissolved in 4% formaldehyde alkalized with 0.1 cc of N NaOH per 10 cc give the best results. Fixation is complete in 6 to 8 hours but periods of fixation as long as 24 hours do not appear to be particularly harmful. When small tissue fragments or thin layers of cells, such as those obtained in tissue culture, are to be fixed, the time of fixation must be reduced, 15 to 30 minutes sufficing in the latter case if the plasma clot has been removed. After fixation, the tissues can be immediately dehydrated in varying strengths of alcohol and imbedded in paraffin or paraffin mixtures. If the tissues are allowed to stand in 70 to 80% alcohol for periods of weeks or months, they darken considerably and harden somewhat, and this has been found to interfere, to some extent, with certain of the staining reactions.

Tissue blocks, prepared as outlined, section easily, and may be cut at thicknesses of

* This research was begun in the laboratories of Pathology and Bacteriology of the Rockefeller Institute for Medical Research.

† Fellow of the International Cancer Research Foundation.

¹ Baker, J. R., *Cytological Technique*, London, Methuen, 1933, p. 131.

have been well shown in such preparations. Red cells, however, appear badly distorted in the larger vessels although they are rather well preserved in the capillaries and arterioles. This deleterious effect is probably not of osmotic origin as it occurs when iso-osmotic solutions of hydroxybenzene-formol are used as well as when 85% NaCl is added to such solutions. Another undesirable feature of these fixatives is that when tissue blocks remain in them sufficiently long to allow penetration and fixation throughout the block, the cells near the surface are over fixed. This over fixed zone is however not very thick when the tissue has been fixed 6 to 8 hours and does not, as a rule interfere with

the study of the tissue

Summary Methods for the fixation of the cell cytoplasm employing pyrogallol or resorcinol in neutralized formaldehyde solution are outlined. Tissue pieces fixed with these solutions have in general shown few signs of distortion and the protein cytoplasmic elements of the cells have appeared well preserved, such structures as mitochondria secretion granules and some of the specific types of granulation being well maintained. The advantages of these methods lie in the simplicity and rapidity of the procedures, the ease with which the tissue can be sectioned at a thickness of 1 to 2 micra, and the generally satisfactory results obtained.

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Antipyridoxine Activity of 2, 4-Dimethyl-3-Hydroxy-5-Hydroxymethylpyridine in the Chick

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In the course of investigations on the vitamin activity of pyridoxine analogs it was discovered that 2,4-dimethyl-3-hydroxy-5-hydroxymethylpyridine had very strong antipyridoxine properties. Growth and survival of pyridoxine-deficient chicks in curative assays were used as the criteria of response. This substance a desoxypyridoxine, had been studied in pyridoxine-deficient rats by Moller¹ and associates and by Unna,² both of whom found no pyridoxine activity. No indication of antipyridoxine activity was reported by these investigators.

Day-old female single comb White Leghorn chicks were fed a commercial starting ration for 3 days and then were given a purified diet (Table I) deficient in pyridoxine. After 6 days on this deficient diet when many chicks were beginning to lose weight

the chicks were divided into groups of 7 selected in such a way that all groups were as nearly alike as possible in weights of the individual chicks. The test substance was given in single oral doses to each chick on the 11th day of age and on the following 3 alternate days. Three or more dose levels of pyridoxine in the range from 0 to 24 μg per dose were given in each assay to establish the standard curve of response for that assay. New standard solutions of pyridoxine and of desoxypyridoxine were prepared in water for each assay and kept under toluene in a refrigerator for the 6-day dosing period. The chicks were weighed before each dose and on the second day after the last dose was given. The pyridoxine-deficient diet was fed to all groups during the assay period.

Two 100 μg doses of desoxypyridoxine were fatal to pyridoxine-deficient chicks even when each dose was preceded by a 16 μg dose of pyridoxine (Table II). Furthermore, a level of the analog as low as 16 μg per

¹Moller E F, Zimra O, Jung F and Moll, Th. *Naturwiss* 1939 27, 228

²Unna K. *Proc Soc Exp Biol and Med*, 1940 43, 122

tions Their reaction to various staining procedures is, however, rather different The Altmann-Bensley method gives the best results for mitochondria The tissue slices should not be treated with potassium permanganate and oxalic acid, and, although good results are obtained if potassium dichromate is employed, somewhat clearer staining is produced by omitting this step Routine hematoxylin and eosin methods, Schleicher's modification of Mallory-Heidenhain connective tissue stain, and Pappenheim's methyl green-pyronin method give good results following resorcinol-formol fixation However, although the nuclei and cytoplasmic ground substance stain well with iron-hematoxylin, the mitochondria do not show up distinctly even after the tissue has been allowed to stand in alcohol until it has darkened

Discussion Some investigation of the action of these hydroxybenzene-formol mixtures has been carried out, and the results warrant terse mention here Although the pH of any fixative is of utmost importance where the cytoplasm is concerned, this aspect is easily adjusted in the solutions outlined above, for, with the addition of NaOH, the weakly acidic pyrogallol and resorcinol form buffer systems The point of greatest buffering power of these systems is in the neighborhood of pH 9, but with the relatively high concentrations used for fixation, 7% pyrogallol for instance, increasing the concentration of NaOH from 0.1 N to 0.2 N only increases the pH from 7.4 to 7.7 and the latter is not excessive for tissue fixation

The effect of these solutions upon cytoplasmic extracts is also of some interest Although neutralized formaldehyde does not cause precipitation of the proteins in such extracts and even protects them from subsequent precipitation with alcohol in concentrations of 50% or less, the hydroxybenzene-formol mixtures cause an immediate and heavy precipitation of the protein components When purified preparations of mitochondria are so treated, the particles agglutinate, but microscopic examination reveals that the individual elements are not appreciably altered morphologically That they have been chemically modified, however, is evidenced by the fact

that after treatment they are insoluble in NaOH even in concentrations of 0.5 N whereas untreated mitochondria dissolve readily in solutions of NaOH that exceed 0.05 N

The reaction of cells in tissue culture was studied by the method of dark-ground illumination as given by Strangeways and Canti.³ As the fixing solutions were drawn across the cells, the cell boundaries became sharply demarcated and, although a few of the finer processes were seen to retract in general the original outlines of the cells were well maintained The ground substance of the cells became much more brilliantly illuminated, but no discrete particles of precipitated protein nor obvious precipitation patterns appeared The perinuclear refractile bodies, which probably represent the Golgi apparatus,⁴ showed little or no tendency to fuse, and apparently were essentially unaltered as they dissolved completely when 95% alcohol was drawn through the chamber The mitochondria became less noticeable because of the increased density of the illumination of the ground substance, but careful inspection revealed no evidence of change in their size or shape either with fixation or with subsequent treatment with alcohol and ether No alteration of size or shape of the nuclei or nucleoli was noted, although the same general increase in density of illumination that was observed in the cytoplasm was also seen in these structures In general, then, direct observations of tissue culture cells during fixation and subsequent treatment with alcohol and ether substantiated the impression gained from a study of sectioned material that these fixatives distort the cell very little and preserve the mitochondria very well

The results obtained with these fixing solutions have been especially satisfactory because, in addition to the preservation of cytoplasmic detail, such structures as brush borders, terminal bars, elastic membranes in the arterial system, and basement membranes

³ Strangeways, T. S. P., and Canti, R. G. *Quart J Micro Sci*, 1927, **71**, 1

⁴ Porter, K. R., Claude, A., and Fullam, E. F., *J Exp Med*, 1945, **81**, 233

TABLE III

Antipyridoxine Activity of Desoxypyridoxine as Measured in 9 day Curative Assays with Chicks Receiving a Pyridoxine Deficient Diet

Oral supplement*		Survival of chicks, alive/total	Wt gain per chick, g	Loss in pyridoxine activity, μ g	Ratio of inhibition, analog vit
Pyridoxine hydrochloride, μ g	Desoxypyridoxine hydrochloride μ g				
16	32	2/7	15.5	11	3.1
16	16	6/7	20.5	7	2.1
16	8	7/7	18.8	8	1.1
4	—	6/7	14.5		
8	—	6/7	19.5		
16	—	7/7	28.0		
16	16	5/7	23.4	3	5.1
24	16	7/7	30.2	8	2.1
35	50	6/7	21.4	23	2.1
—	—	3/7	7.2		
8	—	6/7	16.1		
12	—	7/7	19.6		
16	—	6/6	32.0		

* Amt given per chick on each of 4 successive alternate days

activity of one part of pyridoxine (Table III)

Previously reported B vitamin antagonists are relatively much weaker antivitamin. For example, only one molecule of thiamine was required to overcome the antithiamine activity of 40 molecules of pyrithiamine in mice,⁴ and a similar ratio was found between riboflavin and isoriboflavin rats.⁵ It therefore

appears that desoxypyridoxine is the most potent of the B vitamin inhibitors as yet discovered.

Summary Bioassays with chicks have shown that 2,4-dimethyl-3-hydroxy-5-hydroxymethylpyridine is a very potent inhibitor of pyridoxine. Under the conditions of these experiments, two molecules of the inhibitor were sufficient to offset the vitamin activity of one molecule of pyridoxine. This ratio was found to hold for suboptimal and optimal amounts of pyridoxine given to pyridoxine-deficient chicks.

⁴ Woolley, D. W., and White, A. G. C., *J. Biol. Chem.*, 1943, **149**, 285.

⁵ Emerson, G. A., and Tishler, M., *Proc. Soc. Exp. Biol. and Med.*, 1944, **55**, 184.

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Adrenolytic and Sympatholytic Actions of Priscol (benzyl-imidazoline)

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Experimentally employed adrenolytic agents include ergotoxine, ergotamine tartrate, yohimbine hydrochloride, various esters of yohimbic acid and the Fourneau Compounds, F933 and F899. In some respects these agents seem to be sympatholytic as well as

adrenolytic. It has been reported¹ that Priscol exerts this dual effect.

The adrenolytic properties of priscol have

¹ Meier, R., and Meyer, R. Th. *Schweiz. Med. Wochschr.* 1941 **71**, 1266.

TABLE I
Pyridoxine Deficient Diet for Chicks

Dextrose (cerealose)	46.5
Casein (vit free)	25.0
Gelatin	10.0
Calcium gluconate	5.0
Salts IV ¹	5.0
KH ₂ PO ₄	1.0
Liver extract "L" ²	2.0
Wheat germ oil	4.5
400D fish liver oil	0.5
Cystine	0.2
Choline	0.2
Inositol	0.1
p-Aminobenzoic acid	0.03
Niacin	0.01
Calcium pantothenate	0.004
Riboflavin	0.002
Thiamine	0.002
Menadione	0.0004
Biotin	0.00004
Total	100.05

¹ Wilson & Co., Inc., Chicago, Ill.

² Hegsted, D. M., Mills, R. C., Elvehjem, C. A., and Hunt, L. B. *J. Biol. Chem.* 1941, **138**, 459

dose also proved fatal to the birds. On the other hand, pyridoxine-deficient chicks which received no supplement suffered less than 60% mortality in the same assays.

That the action of the analog was probably due to interference with pyridoxine activity rather than to some other type of toxicity was indicated by a test on chicks receiving a commercial starting ration. Dose levels of 50, 100, and 200 μ g were given to groups

of 3 six-day-old chicks each and repeated on the two following alternate days. All chicks survived, and no retardation of growth was observed. These chicks, with normal body stores of pyridoxine and an adequate supply of the vitamin in the commercial starting ration, were able to tolerate total dosages of at least 600 μ g of the analog, a quantity more than 6 times as great as the lethal dose to more than half the pyridoxine-deficient chicks.

On the assumption that desoxypyridoxine acted as an antivitamin, further assays were conducted with pyridoxine-deficient chicks on the pyridoxine-deficient diet to estimate the amount of the analog needed to counteract a given amount of pyridoxine (Table III). In these tests the dose of pyridoxine always preceded the dose of the analog by 15 to 30 minutes.

The growth response of each group receiving both compounds was compared with the standard curve of response from graded levels of pyridoxine, and the net pyridoxine activity of each combination was estimated. Subtraction of the observed pyridoxine activity from that theoretically expected on the basis of the level of pyridoxine administered gave the apparent loss in pyridoxine activity occasioned by the dose of desoxypyridoxine. From these last 2 values it can be seen that about 2 parts of the analog counteracted the biological

TABLE II
Activity of Desoxypyridoxine and Pyridoxine in 9-day Curative Tests with Chicks Receiving a Diet Deficient in Pyridoxine

Oral supplement*		Survival of chicks, alive/total	Wt gain per chick, g
Pyridoxine hydrochloride μ g	Desoxypyridoxine hydrochloride μ g		
—	100	0/7	(5 dead after 1st dose) (2 " " 2nd ")
—	—	6/7	3.8
16	—	7/7	36.6
16	100	0/7	(4 dead after 1st dose) (3 " " 2nd ")
—	—	4/7	0.7
16	—	7/7	33.0
—	16	0/6	(3 dead after 1st dose) (2 " " 2nd ") (1 " " 3rd ")
—	—	3/7	—7.4
16	—	6/6	32.0

* Amt given per chick on each of 4 successive alternate days

TABLE III

Antipyridoxine Activity of Desoxypyridoxine as Measured in 9 day Curative Assays with Chicks Receiving a Pyridoxine Deficient Diet

Oral supplement*		Survival of chicks, alive/total	Wt gain per chick, g	Loss in pyridoxine activity, μ g	Ratio of inhibition, analog vit
Pyridoxine hydrochloride, μ g	Desoxypyridoxine hydrochloride, μ g				
16	32	2/7	15.5	11	3.1
16	16	6/7	20.5	7	2.1
16	8	7/7	18.8	8	1.1
4	—	6/7	14.5		
8	—	6/7	19.5		
16	—	7/7	28.0		
16	16	5/7	23.4	3	5.1
24	16	7/7	30.2	8	2.1
35	50	6/7	21.4	23	2.1
—	—	3/7	7.2		
8	—	6/7	16.1		
12	—	7/7	19.6		
16	—	6/6	32.0		

* Amt given per chick on each of 4 successive alternate days

activity of one part of pyridoxine (Table III)

Previously reported B vitamin antagonists are relatively much weaker antivitamin. For example, only one molecule of thiamine was required to overcome the antithiamine activity of 40 molecules of pyriethamine in mice,⁴ and a similar ratio was found between riboflavin and isoriboflavin rats.⁵ It therefore

appears that desoxypyridoxine is the most potent of the B vitamin inhibitors as yet discovered.

Summary Bioassays with chicks have shown that 2,4-dimethyl-3-hydroxy-5-hydroxymethylpyridine is a very potent inhibitor of pyridoxine. Under the conditions of these experiments, two molecules of the inhibitor were sufficient to offset the vitamin activity of one molecule of pyridoxine. This ratio was found to hold for suboptimal and optimal amounts of pyridoxine given to pyridoxine-deficient chicks.

⁴ Woolley, D. W., and White, A. G. C., *J. Biol. Chem.* 1943, 149, 285.

⁵ Emerson, G. A., and Tishler, M., *Proc. Soc. Exp. Biol. and Med.*, 1944, 55, 184.

15248

Adrenolytic and Sympatholytic Actions of Priscol (benzyl-imidazoline)

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Experimentally employed adrenolytic agents include ergotamine, ergotamine tartrate, yohimbine hydrochloride, various esters of yohimbic acid and the Fournau Compounds, F933 and F899. In some respects these agents seem to be sympatholytic as well as

adrenolytic. It has been reported¹ that Priscol exerts this dual effect.

The adrenolytic properties of priscol have

¹ Meier, R., and Meyer, R., *Th. Schweiz. Med. Wochschr.*, 1941, 71, 1266.

TABLE I
Pyridoxine Deficient Diet for Chicks

Dextrose (cerealose)	46.5
Casein (vit free)	25.0
Gelatin	10.0
Calcium gluconate	5.0
Salts IV ³	5.0
KH ₂ PO ₄	1.0
Liver extract "L"	2.0
Wheat germ oil	4.5
400D fish liver oil	0.5
Cystine	0.2
Choline	0.2
Inositol	0.1
p-Aminobenzoic acid	0.03
Niacin	0.01
Calcium pantothenate	0.004
Riboflavin	0.002
Thiamine	0.002
Menadione	0.0004
Biotin	0.00004
Total	100.05

* Wilson & Co., Inc., Chicago, Ill

³ Hegsted, D. M., Mills, R. C., Elvehjem, C. A., and Hart, L. B. *J. Biol. Chem.* 1941, 138, 459

dose also proved fatal to the birds. On the other hand, pyridoxine-deficient chicks which received no supplement suffered less than 60% mortality in the same assays.

That the action of the analog was probably due to interference with pyridoxine activity rather than to some other type of toxicity was indicated by a test on chicks receiving a commercial starting ration. Dose levels of 50, 100, and 200 μ g were given to groups

of 3 six-day-old chicks each and repeated on the two following alternate days. All chicks survived and no retardation of growth was observed. These chicks, with normal body stores of pyridoxine and an adequate supply of the vitamin in the commercial starting ration, were able to tolerate total dosages of at least 600 μ g of the analog, a quantity more than 6 times as great as the lethal dose to more than half the pyridoxine-deficient chicks.

On the assumption that desoxypyridoxine acted as an antivitamin, further assays were conducted with pyridoxine-deficient chicks on the pyridoxine-deficient diet to estimate the amount of the analog needed to counteract a given amount of pyridoxine (Table III). In these tests the dose of pyridoxine always preceded the dose of the analog by 15 to 30 minutes.

The growth response of each group receiving both compounds was compared with the standard curve of response from graded levels of pyridoxine, and the net pyridoxine activity of each combination was estimated. Subtraction of the observed pyridoxine activity from that theoretically expected on the basis of the level of pyridoxine administered gave the apparent loss in pyridoxine activity occasioned by the dose of desoxypyridoxine. From these last 2 values it can be seen that about 2 parts of the analog counteracted the biological

TABLE II
Activity of Desoxypyridoxine and Pyridoxine in 9-day Curative Tests with Chicks Receiving a Diet Deficient in Pyridoxine

Oral supplement*		Survival of chicks, live/total	Wt gain per chick, g
Pyridoxine hydrochloride μ g	Desoxypyridoxine hydrochloride μ g		
—	100	0/7	(5 dead after 1st dose) (2 " " 2nd ")
—	—	6/7	3.8
16	—	7/7	36.6
16	100	0/7	(4 dead after 1st dose) (3 " " 2nd ")
—	—	4/7	0.7
16	—	7/7	33.0
—	16	0/6	(3 dead after 1st dose) (2 " " 2nd ") (1 " " 3rd ")
—	—	3/7	—7.4
16	—	6/6	32.0

* Amt given per chick on each of 4 successive alternate days

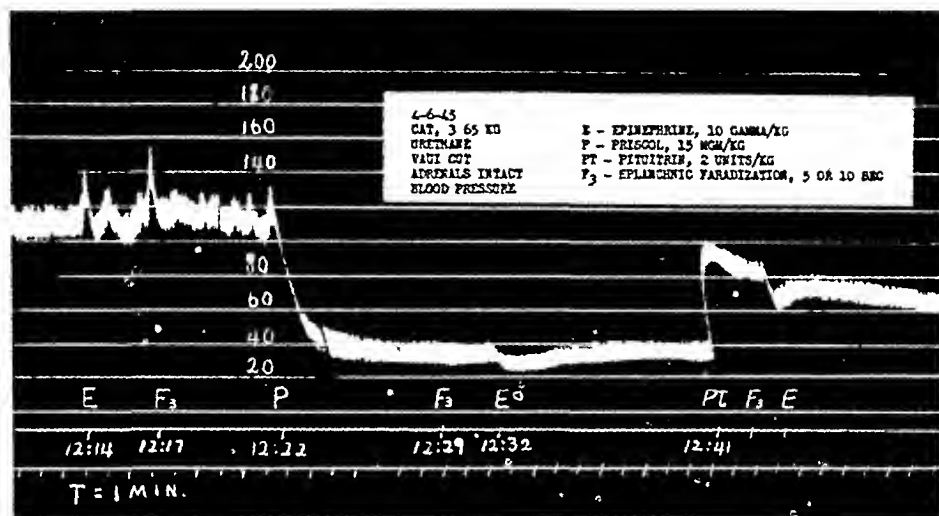


FIG 1

traction of the intestinal musculature under vagal influence might account in part for elevating blood pressure, this could be erroneously interpreted as resulting from splanchnic vasoconstriction. The blood pressure, however, was elevated during splanchnic faradization in the atropinized animal and, therefore, such pressure elevation must have been due only to vasoconstrictor stimulation.

In reference to pressure variations, it seemed to make little difference whether the adrenal glands were intact. In almost every instance faradization of the splanchnic nerves resulted in vasoconstriction, as evidenced by hypertension. Rarely, after prisol paralysis of vasoconstrictors did faradization of the splanchnic nerves produce a slight, transient drop in pressure. This may have been due to splanchnic stimulation of the adrenal gland, resulting in the production of epinephrine, which, in the presence of adequate amounts of prisol, would produce a typical "epinephrine reversal." Such a drop in pressure could also have attended the added relaxation of the intestines resulting from faradization of splanchnic inhibitory fibers to the musculature of the intestine. Since this slight pressure drop occurred also in the absence of the adrenals, it was undoubtedly due chiefly to intestinal relaxation attending stimulation of sympathetic inhibitory fibers in

the splanchnic nerves.

The fact that adrenergic salivation as produced by epinephrine, ephedrine, or cervical sympathetic faradization is depressed by prisol indicates that this drug is a general sympathetic depressant, more potent in respect to some functions than to others. Militating against this generalization, however, is the persistence of mydriatic responses to epinephrine and faradization after prisol in the doses employed. Whether higher doses would nullify pupillary responses is problematic, in any event, sufficient evidence is presented to indicate that prisol varies in its adrenolytic and sympatholytic potencies in the cat in respect to different sympathetic functions. Such variance might prevail in other species, including man.

The antisymphatic properties of prisol suggest the use of this drug in Raynaud's disease as recently reported by Lindquist⁴ and in other conditions of sympathetic predominance, for instance in hypertension of neurogenic origin as postulated by Hevmans⁵ where it should act not symptomatically as a depressor but etiologically as an antagonist to excessive adrenergic stimuli.

⁴ Lindquist T, *Acta Med Scand*, 1943, 113, 83, abstracted in *J A M A*, 1945, 127, 618.

⁵ Hevmans, C, and Bouckaert, J T, *C R Soc Biol* 1931, 106, 471.

been corroborated² in respect to alterations in blood pressure in urethanized cats and it seemed desirable to test the drug's sympatholytic properties. This was done not only in relation to general circulatory responses but also in reference to other functions under control of the sympathetic nervous system.

Methods Adult cats of both sexes were prepared under urethane or DIAL-With-Urethane for carotid cannulation to record arterial pressure by the Anderson⁴ manometer. The vagi were severed. The drugs employed, injected intravenously on a basis of milligrams per kilogram, included epinephrine HCl, priscol HCl, atropine SO₄, ephedrine SO₄ and pituitrin.

In some of our experiments shielded electrodes were attached to either or both sets of splanchnic nerves and the adrenal glands were extirpated or ligated.

The secretory function of the submaxillary gland, as influenced by injection of drugs or faradization of the ipsilateral chorda tympani and cervical sympathetic nerves, was determined after cannulation of Wharton's duct by measuring the salivary flow on a horizontally placed manometer. Pupillary responses were observed and measured directly.

Results Twenty-four experiments were performed and the most significant findings were:

1 Priscol, 5 mg, without exception, produced a drop in blood pressure of 50% or more, pressure gradually returned to normal within an hour or two. Adrenolysis invariably prevailed for vasoconstrictor neural components since an "epinephrine reversal" was consistently obtained during the hypotension caused by priscol. Sympatholysis of vasoconstrictor components, however, did not prevail with this dose of priscol, although it could be produced readily with a 10 to 15 mg dosage (Fig 1).

2 Priscol was both adrenolytic and sympatholytic in respect to sympathetic control

of salivary secretion. The response to epinephrine was usually diminished or lost before the response to faradization of the cervical sympathetic nerve had been completely abolished. The required dose of priscol varied from 1 to 20 mg for adrenolysis and from 5 to 20 mg for sympatholysis. No consistent correlation could be established between the sympathetic inhibition of salivation and the loss of vasoconstrictor control of blood pressure effected with equal doses of priscol. These dose ranges were greater than those suggested in our preliminary report.²

3 Atropine, 0.2 mg, did not interfere with the antisymphathetic actions of priscol and neither did pituitrin (Fig 1).

4 Priscol also inhibited the salivary response that normally is invoked by ephedrine.

5 Priscol initiated salivation, but in only 2 cases and only after high dosage, 12 to 30 mg.

6 The pupil responded well after priscol by dilating in response to administration of epinephrine or to faradization of the cervical sympathetic nerve. In some of our experiments, priscol caused constriction of the pupil, this occurred with a dose of 3 to 5 mg.

Discussion With these experiments it has been confirmed that priscol possesses the dual antisymphathetic action of other known sympathetic-blocking agents, namely, the adrenolytic and sympatholytic actions which characterize such substances. This is most obvious in the responses of the vascular system after priscol epinephrine reversal precedes nullification of splanchnic vasoconstriction to faradization.

In order to rule out the severe hypotension induced by priscol as the cause of splanchnic nerve paralysis, the pressure was raised by pituitrin (Fig 1), during this elevation of tension adrenolysis and sympatholysis still prevailed.

Atropine was employed for two reasons (1) to determine the effect of the loss of cholinergic control of salivation in relation to priscol's antisymphathetic actions and, (2) to rule out vagal stimulation of the gut by faradization since vagal components may run through splanchnic nerves, in which case con-

² Chess, D., and Younkman, F. F., *Fed. Proc.*, 1945, **4**, 114.

⁴ Anderson, F. F., *J. Lab. and Clin. Med.*, 1941, **26**, 1520.

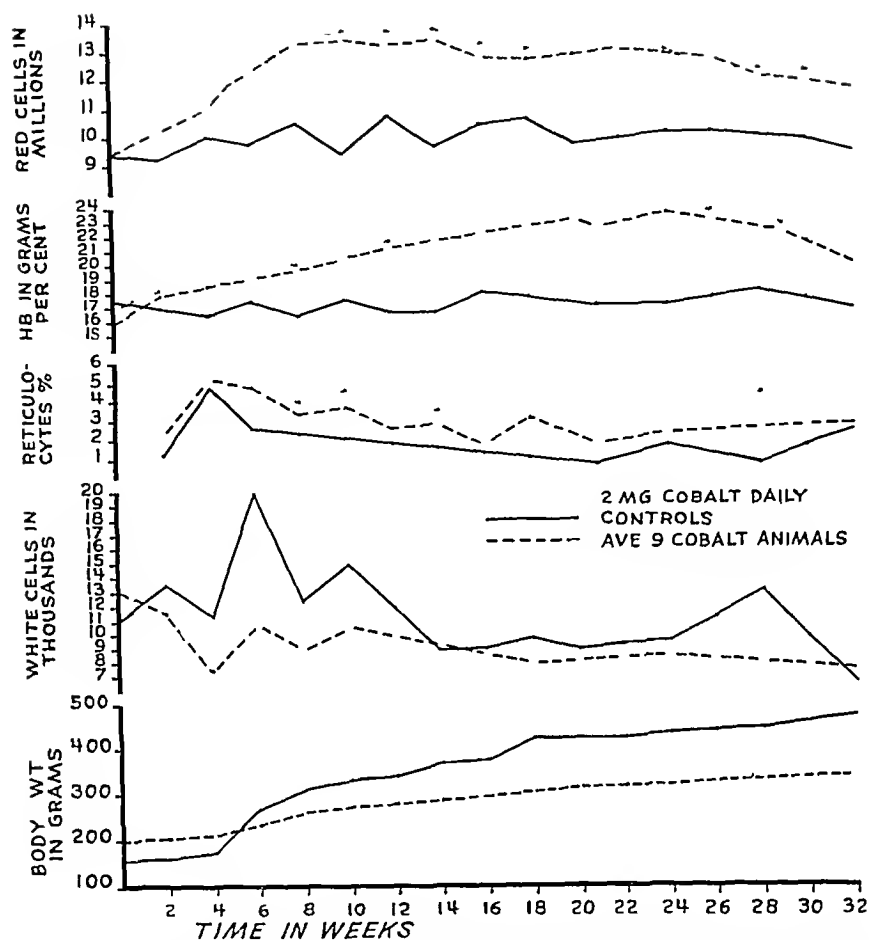


FIG 1

The course of polycythemia produced by cobalt in rats. The dotted line in each of the 3 upper charts shows the maximal values in red cells, hemoglobin, and reticulocytes produced by 2 mg of cobaltous chloride daily.

were determined in the following manner. The dye T-1824 (Evans blue) in a solution of 10 mg per ml was injected into a tail vein in amounts of 15 mg per kg of body weight. Twenty-five minutes \pm 30 seconds were allowed for the dye to mix with the circulating blood. The animals were lightly anaesthetized with ether for one to 1½ minutes prior to drawing 20 ml of blood by heart puncture. A precise amount of dry ammonium and potassium oxalate was used as an anticoagulant. Samples for blood counts were taken from the non-oxalated blood while smears for differential leucocyte and reticulo-

cyte counts were made from the oxalated blood. The mean cell diameters of the red cells were determined by means of a Haden-Hausser erythrocytometer using a fresh solution of 14% sodium oxalate as the diluent. Hematocrit determinations were made and the supernatant plasma was used for plasma volume calculation. A Klett-Summerson Photoelectric colorimeter employing a Wratten filter F-29 was used to determine the dye concentration. In order to limit blood loss to a minimum, the plasma of each animal was diluted from 1:4 to 1:7 with saline and centrifuged immediately prior to reading.

Summary and Conclusions Priscol (benzylimidazoline) is sympatholytic as well as adrenolytic in reference to the sympathetic neural control of blood pressure and salivation. Adrenolysis invariably precedes sympatholysis with any dose that is adequate to produce both effects. These pharmacodynamic actions persist during priscol hypotension, pituitrin hypertension and in the pres-

ence of atropine.

Although sympathetic depression of salivation and vasoconstriction can be produced with variable doses of priscol, up to 15 mg, they are without sympatholytic or adrenolytic effect upon the cervical sympathetic neural components controlling mydriasis, indicating that a differential sympathetic depression characterizes this antisympathetic agent.

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Observations on Cobalt Polycythemia. I. Studies on the Peripheral Blood of Rats *

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The experimental production of polycythemia by the administration of cobalt has been demonstrated by a number of investigators since its discovery by the Waltners.¹⁻⁹ This effect of cobalt has also received application in the treatment of various anemias in both animals and human beings.^{10, 11}

The mechanism of action of cobalt

remains obscure. Several theories have been advanced, however none has been fully agreed upon as a satisfactory explanation by those who have investigated the problem. During the course of some experiments relating to factors influencing various types of anemia, certain new observations concerning the action of cobalt came to our attention.

Male rats of the Sprague-Dawley strain, 4 to 5 months of age, weighing approximately 200 g were given cobaltous chloride, $\text{CoCl}_2 \cdot 6(\text{H}_2\text{O})$, 0.4% solution, by subcutaneous injection in the back and shoulder region 6 times weekly throughout a period of 8 months. Nine animals, divided into 3 groups of 3 each, received 2.5 mg, 5.0 mg and 10.0 mg per kg of body weight respectively. Other animals of the same age, weight, and sex served as controls. The rats were fed dog chow only (Friskies, Albers Milling Co.).

Erythrocyte, reticulocyte, leucocyte and differential counts as well as hemoglobin determinations were made every 2 weeks. Care was taken to draw only the amount of blood necessary for the determinations, the puncture wound in the tail vein being promptly closed after each bleeding. A Haden-Hausser hemoglobinometer (Improved Clinical Model) was used for the hemoglobin determinations.

At the end of 32 weeks, blood volumes

* This work was supported by a grant from the University of Oklahoma School of Medicine.

1 Waltner, K., and Waltner, K., *Klin Wchenschr*, 1929, **8**, 313.

2 Oiten, J. M., Underhill, F. A., Mugridge, E. R., and Lewis, R. C., *Proc Soc Exp Biol and Med*, 1931, **29**, 174.

3 Oiten, J. M., Underhill, F. A., Mugridge, E. R., and Lewis, R. C., *J Biol Chem*, 1932, **99**, 457.

4 Davis, J. L., *Proc Soc Exp Biol and Med*, 1937, **37**, 96.

5 Burton, A. G., and Burton, E. S. G., *Proc Soc Exp Biol and Med*, 1936, **35**, 407.

6 Marschall, P., *Arch ital biol*, 1930, **82**, 112, *Chem Abstr*, 1930, **24**, 3280.

7 Suttler, J., *C R Soc de biol*, 1934, **116**, 994.

8 Stuebe, F. J., and Elvehjem, C. A., *J Biol Chem*, 1933, **99**, 473.

9 Davis, J. L., McCullough, A. W., and Rigdon, R. H., *J Lab and Clin Med*, 1945, **30**, 327.

10 Kleinberg, W., Gordon, A. S., and Charappei, H. A., *Proc Soc Exp Biol and Med*, 1939, **42**, 119.

11 Kato, K., *J Ped*, 1937, **11**, 385.

regular 2-week intervals. It is apparent that the response of reticulocytes, red cells, and hemoglobin is influenced slightly by the dosage of cobalt but that the average figure for the 3 groups is close to that elicited by the maximum dose of 100 mg per kg of body weight. The curves for body weight and leucocyte count similarly show no marked differences as affected by the 3 doses given. While the experimental animals failed to gain as much weight as the controls, no other deleterious effects were noted, which indicates a low systemic toxicity from cobaltous chloride in adult animals in the dosage administered.

Table I presents the numerical data for each animal relative to the blood volume at the end of 32 weeks. The striking increase in total red cell mass and the slight but consistently lower plasma volumes are worthy of note.

Table II contains data derived from cell counts, measurements of the mean cell diameter, hematocrit and hemoglobin content. The cell size, cell volume, cell thickness, absolute hemoglobin content and hemoglobin concentration were calculated according to Wintrobe¹². Regarding the size, shape and hemoglobin content of erythrocytes from animals with cobalt polycythemia the following facts

have come to light as a result of this study: 1 Although the mean cell diameter remains normal the cell volume increases by about one-third, a change which is proportionate to the increase in cell thickness. 2 The absolute amount of hemoglobin per red cell remains unchanged.

The blood volume per kilogram of body weight of these animals compares favorably with that found in the more extreme cases of polycythemia vera in man.¹³

Detailed histopathologic studies are in progress as well as further work on the components of red cells and plasma.

Conclusions 1 Injections of cobaltous chloride continued over a period of 8 months without apparent serious toxicity has resulted in persistent polycythemia during this period of a degree comparable to that occurring in polycythemia vera in man. 2 The average increases in blood volume and total erythrocyte mass as compared with controls was 80% and 192% respectively, due entirely to increase in erythrocytes since plasma volume decreased 16% on the average. 3 The erythrocyte of polycythemic animals as compared with the normal increased in volume 41% due almost entirely to an increase in the thickness of the cell. The hemoglobin content remains essentially unchanged.

¹² Wintrobe, M. M., *Clinical Hematology*, Lea & Febiger, 1942.

¹³ Gibson, J. B., 2d, Harris, A. W., and Swigert, V. W., *J. Clin. Invest.*, 1939, **18**, 621.

15250

Influence of Dimethylaminoethylbenzhydryl Ether Hydrochloride Upon Histamine Flare Reactions

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The alleviation of histamine induced anaphylaxis in guinea pigs has been described following the use of a number of benzhydryl alkamine ethers of the general formula, where R represents a carbon chain carrying a primary, secondary or, in most cases, a

tertiary nitrogen atom.^{1,2,3} In one of the

¹ Loew, E. R., Kruser, M. E., and Moore, V., *J. Pharm. and Exp. Therap.*, 1945, **83**, 120.

² Loew, E. R., and Kruser, M. E. *Proc. Soc. Exp. Biol. and Med.*, 1945, **58**, 235.

³ Shup, E. A., personal communication.

STUDIES ON PERIPHERAL BLOOD OF RATS

TABLE I
Blood Values of Cobalt Injected Rats

Rat No	Body wt, g	Hematocrit	Blood vol, cc	Blood vol, cc/kg	Plasma vol, cc	Plasma vol, cc/kg	Red cell vol, cc	Red cell vol, cc/kg	Blood/body wt	Red cells/body wt	Plasma/body wt
1	340	76	44.46	130.8	10.67	31.4	33.79	99.4	13.07	9.94	3.14
2	345	Data lost									
3	360		49.51	137.6	13.80	38.6	35.65	99.0	13.76	9.90	3.86
4	264	81	43.00	162.8	8.17	30.9	34.83	131.9	16.28	13.19	3.09
5	343	65	41.60	121.2	14.56	42.4	27.04	78.9	12.12	7.88	4.24
6	338	75	59.24	175.2	14.81	43.8	44.43	131.4	17.52	13.14	4.38
7	296	76	47.84	161.6	11.48	38.7	36.36	122.9	16.16	12.29	3.87
8	332	77	54.04	162.7	12.43	37.4	41.61	125.3	16.27	12.53	3.74
9	321	74	44.56	138.8	11.58	36.0	32.98	102.8	13.88	10.28	3.60
Avg	324	74.5	48.03	148.8	12.19	37.4	35.83	111.4	14.88	11.14	3.74
Controls											
10	457	43.5	34.12	74.6	19.38	42.4	14.74	32.2	7.46	3.22	4.24
11	487	48	38.00	78.0	19.76	40.5	18.24	37.4	7.79	3.74	4.05
12	315	49	29.6	94.0	15.1	47.9	14.5	46.0	9.40	4.60	4.79
13	312	44	26.4	84.6	14.8	47.5	11.6	37.1	8.46	3.71	4.75
Avg	393	46.1	32.03	82.8	17.26	44.5	14.79	38.2	8.28	3.82	4.45
Difference between controls and experimental animals											
	28.4	16.00	66.0	5.07	7.1	21.04	73.2	6.60	7.32	7.1	
% increase or decrease experimental over controls											
	+61	+50	+80	-29.3	-16	+142	+192	+80	+192	-16	

Data derived from hematocrit and plasma volume determinations at the end of 32 weeks of cobalt injections. Rats 1, 2, and 3 received 0.5 mg cobalt daily, rats 4, 5, and 6 received 1.0 mg cobalt daily, rats 7, 8, and 9 received 2.0 mg cobalt daily.

TABLE II
Data Relative to Shape, Size, and Hemoglobin Content of Red Cells of Rats After Injections of Cobalt

	Controls	Cobalt	% increase
Mean cell diameter in μ	6.45	6.55	—
Mean red cell volume in μ^3	47.0	66.5	41.0
Mean red cell thickness in μ	1.44	1.97	36.8
Mean red cell hemoglobin in $\mu\mu\text{g}$	17.0	17.0	—
Mean red cell hemoglobin conc in %	36.4	26.5	-27.4

Data relative to the red cells of rats injected with cobalt. The mean corpuscular volume and mean corpuscular thickness are derived from red cell counts, hematocrit determinations and mean cell diameters. The hemoglobin values are calculated from hemoglobin determinations, red cell counts and total red cell mass.

Calibration curves were made on rat plasma containing known amounts of T-1824 and also diluted 1:4 to 1:7. Dilution of rat plasma with as much as 7 parts of saline did not alter the absorption characteristics of T-1824 as determined by the photoelectric colorimeter. At the time the blood volume

determinations were made, blood counts also were made using blood taken from a tail vein and from the heart of each animal. The differences between the two counts for each animal were within the tolerated limits of technical error.

Fig. 1 summarizes the data obtained at

TABLE I
Erythema and Wheel Reactions to the Intravenous Administration of Dimethylaminoethyl Benzhydryl Ether Hydrochloride

No subjects	Dosage (mg/daily)	Duration treatment (weeks)	Degree of Reaction (% of control)†			
			Erythema		Wheel	
			5 mm	10 mm	5 mm	10 mm
10	0	—	100	100	100	100
5	150	0.6	105	63	63	75
5	300	2.0	4	37	3	7
3	400	2.3	24	33	4	8
6	—	0.5†	138	155	185	168
2*	—	1.0†	493	300	104	104

* Both of these patients had received treatment for more than 11 weeks, and at the 400 mg dosage level for not less than 2 weeks

† Length of time since treatment was discontinued

‡ Figures represent averages for the number of subjects indicated

to develop, even on long continued administration, and the clinical results thus far attained,⁴⁻¹⁰ warrant further extensive clinical trial of the drug in a wide variety of allergic conditions

Summary Dimethylaminoethylbenzhydryl

ether hydrochloride orally administered in sufficient amounts is capable of completely suppressing the sensitivity of the skin to histamine, at least as expressed by the wheel and flare reaction. In limited trials, this effect appears to have widespread clinical application

15251

The Reputed Antipyretic Action of Camphor *

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Many current textbooks of pharmacology contain statements to the effect that camphor is antipyretic and useful in the symptomatic treatment of fevers. Thus for example, in Sollmann's treatise¹ we find, "Camphor Spirit is a household remedy in colds, bronchitis, etc. It is thought to be somewhat expectorant, diaphoretic and antipyretic (Binz, 1875 and 1877)." In older books, published 100 years or more ago, less cautious statements

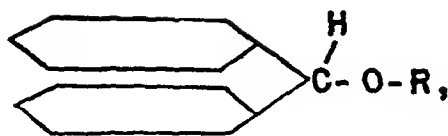
are made, for there the virtues of camphor as an antipyretic are panegyrised. Camphor was first used in China and brought to Europe by the Arabians.² Its introduction as an antipyretic seems to have been based upon at least two properties, first that it has some antidiarrhetic activity and secondly that spirit of camphor, applied to the skin, has a cooling effect. There has been no recent work done to check these ancient conceptions of the antipyretic value of camphor.

In the work described in this report, some 500 animals, including albino rats, guinea pigs, rabbits and cats, were rendered febrile by the intramuscular injection of peptone (B.D.H.) in a dose of 1 g per kilo body

* This research was aided financially by a grant from the Committee of Scientific Research, Queen's University. A report of the work was presented at the annual meeting of the American Society for Pharmacology and Experimental Therapeutics, Inc., Atlantic City, March, 1946.

¹ Sollmann, T., *A Manual of Pharmacology*, 6th ed., Saunders, London, 1942.

² Fleckiger, F. A., and Hanbury, D., *Pharmacographia*, Macmillan London, 1879.



most potent of these preparations, the R grouping is beta-dimethylaminoethyl¹²

The present experiments have been conducted to determine the degree to which histamine flare reactions in the skin can be influenced by the oral administration of dimethylaminoethyl benzhydryl ether hydrochloride* to human subjects

Methods and Materials Human beings who were not known to have any allergic disease or other condition associated with a recognizable disturbance of the autonomic nervous system were subjected to histamine flare tests before, during, and after the oral administration of varied amounts of dimethylaminoethyl benzhydryl ether hydrochloride

To examine skin sensitivity to histamine, a drop of 1% aqueous histamine solution and a drop of physiological saline for control purposes were each applied to the volar surface of the forearm. A slight depression of the skin under each drop was then made by pressure from a sterilized ordinary pin, care being taken not to cause any bleeding. After 5 minutes, the drops were gently wiped away, and the size and shape of the wheal and erythema were outlined with ink. This out-

line was accurately transferred for permanent record by the use of transparent paper and india ink. At the end of 10 minutes a similar reading and recording of the wheal and erythema was made. The size of the wheal and of the erythema were approximated by multiplying the diameters at right angles by each other. The values thus obtained in each subject in the fore period were arbitrarily taken as 100%, and all subsequent values computed as fractions or multiples thereof.

Results The data (Table I) reveal that a daily dose of 150 mg of dimethylaminoethylbenzhydryl ether hydrochloride was capable of a definite reduction in the sensitivity of the skin to histamine, and that 300 mg daily might effect a maximum response if sufficiently long continued. A diminution in both wheal and erythema has been observed after 3 days of treatment with 150 mg daily. A complete disappearance of both wheal and erythema has been noted in some cases after 10 days of treatment with 300 mg. The continued administration of the drug, at least for periods up to 18 weeks apparently does not alter the striking anti-histamine action. As a rule a maximum effect can be attained with a dose of 300 mg daily, although this may not be reached quite so promptly as when 400 mg are given.

When the drug is discontinued, the response to histamine is accentuated as compared with the control period. In one instance, the "release" phase was associated with a flare and wheal that were approximately 500% of the original. In every case, this phasic effect is seen, but the response to histamine usually returns to normal within a few days after dimethylaminoethyl benzhydryl ether hydrochloride has been stopped. In no previously normal subject was this increased response to histamine associated with any clinical allergic manifestation.

Discussion Undoubtedly the anti-histamine action of dimethylaminoethylbenzhydryl ether hydrochloride accounts for the clinical success attendant upon its application to several types of skin condition.^{4,5} The high therapeutic index, the failure for tolerance

* Generous supplies of this material under the name, Benadryl, have been made available through the courtesy of Dr E A Sharp, of Pirke, Davis & Co, Detroit, Mich.

⁴ Curtis, A C, and Owens, B B, *Univ Hosp Bull*, Ann Arbor, 1945, 11, 25

⁵ O'Leary, P A, and Friber, E M, *Proc Staff Meetings of the Mayo Clinic*, 1945, 20, 429

⁶ McElhin, T W, and Horton, B T, *Proc Staff Meetings of the Mayo Clinic*, 1945, 20, 417

⁷ Koelsche, G A, Friedman, L E, and Cruijer, H M, *Proc Staff Meetings of the Mayo Clinic*, 1945, 20, 432

⁸ Williams, H L, *Proc Staff Meetings of the Mayo Clinic*, 1945, 20, 434

⁹ Logan, G B, *Proc Staff Meetings of the Mayo Clinic*, 1945, 20, 436

¹⁰ McGivach, T H, Elias, H and Boyd, L J, *J Lab and Clin Med*, in press

TABLE I
Erythema and Wheal Reactions to the Intracutaneous Administration of Dimethylaminoethyl Benzhydryl Ether Hydrochloride

No subjects	Dosage (mg/daily)	Duration treatment (weeks)	Degree of Reaction (% of control)†			
			Erythema		Wheal	
			5 min	10 min	5 min	10 min
10	0	—	100	100	100	100
5	150	0.6	105	63	63	75
5	300	2.0	4	37	3	7
3	400	2.3	24	33	4	8
6	—	0.5†	138	155	185	168
2*	—	1.0†	493	300	104	104

* Both of these patients had received treatment for more than 11 weeks, and at the 400 mg dosage level for not less than 2 weeks

† Length of time since treatment was discontinued

‡ Figures represent averages for the number of subjects indicated

to develop, even on long continued administration, and the clinical results thus far attained,⁴⁻¹⁰ warrant further extensive clinical trial of the drug in a wide variety of allergic conditions

Summary Dimethylaminoethylbenzhydryl

ether hydrochloride orally administered in sufficient amounts is capable of completely suppressing the sensitivity of the skin to histamine, at least as expressed by the wheal and flare reaction. In limited trials, this effect appears to have widespread clinical application

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The Reputed Antipyretic Action of Camphor*

ELDON M. BOYD, AND KATHLEEN G. W. SEYMOUR

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Many current textbooks of pharmacology contain statements to the effect that camphor is antipyretic and useful in the symptomatic treatment of fevers. Thus for example, in Sollmann's treatise¹ we find, "Camphor Spirit is a household remedy in colds, bronchitis, etc. It is thought to be somewhat expectorant, diaphoretic and antipyretic (Binz, 1875 and 1877)." In older books, published 100 years or more ago, less cautious statements

are made, for there the virtues of camphor as an antipyretic are panegyricised. Camphor was first used in China and brought to Europe by the Arabians.² Its introduction as an antipyretic seems to have been based upon at least two properties, first that it has some antidiarrheic activity and secondly that spirit of camphor, applied to the skin, has a cooling effect. There has been no recent work done to check these ancient conceptions of the antipyretic value of camphor.

In the work described in this report, some 500 animals, including albino rats, guinea pigs, rabbits and cats, were rendered febrile by the intramuscular injection of peptone (BDH) in a dose of 1 g per kilo body

* This research was aided financially by a grant from the Committee of Scientific Research, Queen's University. A report of the work was presented at the annual meeting of the American Society for Pharmacology and Experimental Therapeutics, Inc., Atlantic City, March, 1946.

¹ Sollmann, T., *1 Manual of Pharmacology*, 6th ed., Saunders, London, 1942.

² Fluckiger, F. A., and Hanbury, D., *Pharmacographia*, Macmillan London, 1879.

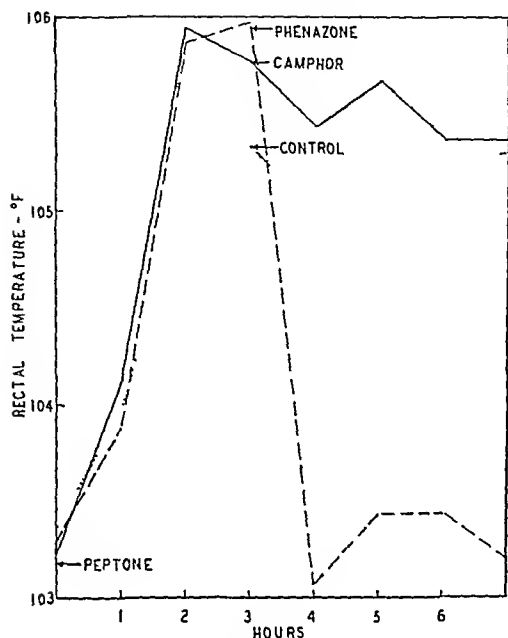


Fig 1

The effect of phenazone and of camphor upon peptone induced fever in rabbits

weight. In the first experiment, 66 rabbits were divided into 6 groups. Rabbits in 3 of the groups were injected with peptone while rabbits in the other 3 groups received no peptone. Rectal temperatures were taken before the injection of peptone and at hourly intervals thereafter, the latter in all groups of animals. Three hours after the injection of peptone, when fever was at its height, rabbits in one group were given an intramuscular injection of camphor in oil at a dose of 100 mg per kilo body weight, at the same time the second group of febrile rabbits received phenazone B P, (antipyrine, USP) at a dose of 200 mg per kilo body weight, the third group of febrile rabbits received no further treatment and acted as controls. The remaining 3 groups of afebrile rabbits, which received no peptone, were treated in a corresponding manner at the end of the third hour of the experiment, *i e* one group received phenazone, the second camphor and the third no further treatment.

There was no appreciable change, over the period of 7 hours of the experiment, in the mean rectal temperature of rabbits receiving

no peptone, no camphor and no phenazone. Neither did phenazone nor camphor have any significant effect upon the mean rectal temperature of afebrile rabbits. There were distinct changes in rectal temperature of rabbits injected with peptone and the means of these have been plotted in Fig 1. The control febrile rabbits developed a mean rectal temperature which varied between 105° F and 106° F. The injection of phenazone rapidly reduced the febrile temperature to normal. The injection of camphor, however, had little effect upon febrile temperature.

This experiment demonstrated that camphor, in the dose used, was not antipyretic under conditions in which phenazone had a marked antipyretic effect. There was the possibility, however, that camphor might have been antipyretic in smaller or larger doses than that used. To investigate this, a second experiment was performed in which 6 groups of 12 rabbits each were given peptone and then respectively, 0, 1, 10, 50, 100, and 500 mg per kilo body weight of camphor dissolved in oil and injected intramuscularly. In this entire series of animals, camphor had no consistent effect upon the febrile temperature.

Thus far, our experiments proved that camphor was not antipyretic when injected into rabbits with a fever induced by injection of peptone. We next considered the possibility that camphor might be antipyretic in other species of animals. Accordingly, the technique used in the second experiment, just described, was applied to 72 albino rats, 66 guinea pigs and 80 cats, each divided into 6 groups, injected with peptone and the respective doses of camphor. The 500 mg per kilo dose of camphor produced convulsions in cats and was discontinued after 5 trials. In no instance was there any evidence that camphor had any appreciable antipyretic activity.

In these 3 experiments, camphor was not found to be antipyretic in animals with a fever induced by injecting peptone. There was the possibility that it might be antipyretic towards fever induced by pyrogens other than peptone. From time to time, we

had available animals with a natural fever due, usually, to infection and abscess formation at the point where peptone had been injected. This group of animals included 9 rabbits, 6 guinea pigs, 8 albino rats and 6 cats. We made no systematic study of these but injected them with various doses of camphor in oil. In the entire group of 29 animals, 24 or 83% showed no change or increased fever after injecting camphor, in 4 animals the temperature fell toward normal and 1 animal died.

Finally, we considered the possibility that camphor given by stomach tube in the form of Spirit of Camphor, B P, might be antipyretic. Six groups of 13 rabbits each were injected with peptone and 3 hours later given respectively 0, 0.01, 0.1, 0.5, 1.0, and 5.0 ml of Spirit of Camphor by stomach tube, washed down with water. Again camphor failed to exhibit any antipyretic activity.

These 5 experiments convinced us that camphor has little or no antipyretic activity and that statements to the effect that the internal use of camphor is "supposed" or "considered" to have some antipyretic value should be deleted from textbooks, thus in some measure, even though small, lightening the burden of information which the medical student, physician and pharmacologist have to learn.

Summary Camphor was administered parenterally and by stomach tube, in a wide range of doses, to some 500 animals, including albino rats, guinea pigs, rabbits and cats with a fever induced by previous injection of peptone and, in a few instances, fever from infection. Under these conditions, phenazone was found to be antipyretic in rabbits, but no evidence was obtained that camphor possessed antipyretic properties, at least to any marked degree.

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Blood Chemical Changes Following Intravenous Administration of a Casein Hydrolysate to Human Subjects *

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To determine the criteria for the proper administration of Parenamine,[‡] one of the commercially available casein hydrolysates which are used parenterally, and as a part of an investigation of its clinical effective-

ness, a study was made of some of the blood chemical changes that occurred when this preparation was injected intravenously. The clinical studies have been reported elsewhere.¹

Forty-five g of Parenamine (equivalent to 6 g of nitrogen) dissolved in 1000 cc of distilled water were injected intravenously into 30 hospital control subjects at a commonly used clinical speed of 300 to 350 cc per hour. The injections were made in the morning, breakfast having been withheld. Blood samples were drawn at the beginning, middle and end of injections and at 1 and 2 hours after the injection was completed. Plasma amino acid nitrogen concentrations were de-

* Aided by a grant from Frederick Stearns and Company, Detroit, Mich.

[†] Abbott Fellow in Surgery, Northwestern University Medical School.

[‡] Kindly furnished by Frederick Stearns and Company, Detroit, Mich. Parenamine is an acid hydrolysate of casein, fortified with 1% D, L tryptophane. It is put up in 15% solutions in bottles containing 100 cc. The method of preparation of Parenamine is essentially that described by Sahyun.⁷ An analysis of the distribution of the amino acids in the preparation has been reported by Block and Bolling.⁸

¹ Kozoll, Donald D., Hoffman, William S., and Meyer, Karl A., *Arch. Surg.*, 1945, **51**, 59.

TABLE I
Blood Chemical Changes Following Intravenous Injection of 45 G of Casein Hydrolysate

Blood Constituent Analyzed	No of Cases	Mean Levels During and After Injection					Modal Time of Max Change
		At Start of Inj	Middle of Inj	End of Inj	1 Hr After	2 Hr After	
Amino Acid N, mg N per 100 cc Plasma	30	51 +15* 2584†	94 +14 61112†	92 +12 72112†	70 +12 4690†	60 +11 4080†	Middle of Inj +49 +17
Urea N, mg, N per 100 cc blood	19	115	136	157	174	173	1 Hr after Inj +66
Inorganic Phosphate, mg P per 100 cc Plasma	18	36	31	35	39	40	Middle of Inj -05
CO ₂ Combining Power, cc per 100 cc Plasma	13	63	62	61	62	64	End of Inj -4

* Numbers after ± represent standard deviation
† Range

terminated by the naphthoquinone method adapted to the photoelectric colorimeter by one of us (W S H),² inorganic phosphate photoelectrically by Fiske and Subbarow's method,³ urea by the method of Hoffman and Osgood⁴ and CO₂ combining power by the method of Van Slyke,⁵ and glucose by the method of Hoffman⁶

The results are summarized in Table I. The initial plasma amino acid N levels ranged between 25 and 84 mg per 100 cc averaging 51 with a standard deviation of ± 15 . These values are slightly higher than those obtained with the ninhydrin method.⁹ The amino acid N levels rose during the injection reaching a maximum in the majority of cases in the middle of the injection, in the remainder at the end of the injection. Thus the rate of removal of amino acids from the blood stream eventually became faster than the 15 g per hour of injection. The average maximal rise was 49 mg per 100 cc. At the end of 2 hours the amino acid N concentration had returned almost to the base level. The amino acid curves on the whole agreed with those found by Landesman and Weinstein¹⁰ for intravenous injection of Amigen.

The blood urea N concentration began to rise slowly during the injection reaching a maximal value in 11 cases at 1 hour after the injection, and in 8 cases at 2 hours after the injection. The average maximal urea N concentration was 174 mg per 100 cc. The highest maximal concentration was 271 mg

² Hoffman, William S, *Am J Clin Path*, 1945, 15, 57

³ Fiske, C H, and Subbarow, Y, *J Biol Chem*, 1925, 66, 375

⁴ Hoffman, William S, and Osgood Bess, *J Lab and Clin Med*, 1940, 25, 862

⁵ Van Slyke, D D *J Biol Chem* 1917, 30, 347

⁶ Hoffman, William S, *J Biol Chem*, 1937, 120, 51

⁷ Shihyun, M, *Proc Soc Exp Biol and Med*, 1941, 48, 14

⁸ Block, R J and Bolling, D, *Am J Pharm*, 1944 116, 368

⁹ Woodruff, C W, and Min, L B, *J Biol Chem*, 1945, 157, 93

¹⁰ Landesman, R, and Weinstein, V A, S G and O, 1945, 75, 300

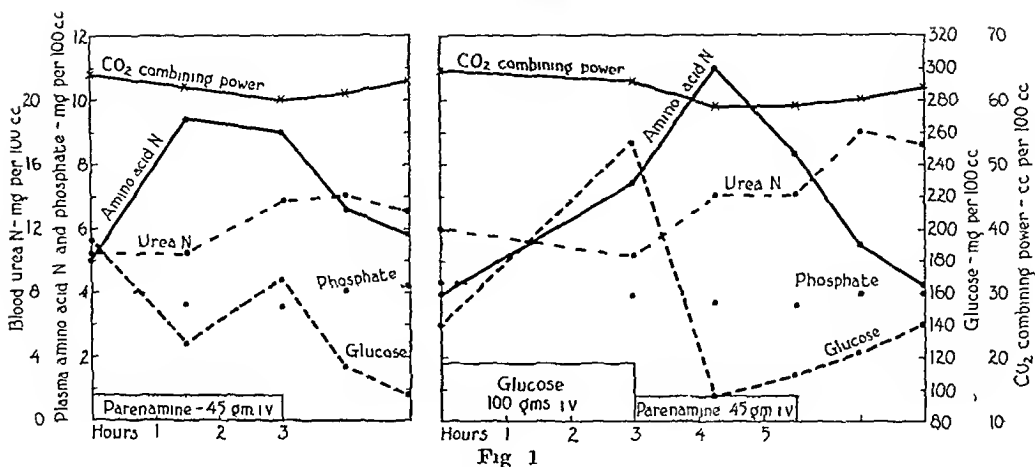


Fig 1

per 100 cc, this was in a patient whose initial level was 18.3 mg per 100 cc and who therefore may have had an early renal insufficiency. With one other exception, the remaining subjects showed maximal urea concentrations within the limits of normal such as might be found in patients on a high protein diet. Urea clearance determinations made in the successive intervals in 8 cases were too variable to indicate any direction of change, but at least they gave no clear evidence that the rise in blood urea concentration was due to a diminished rate of renal excretion.

A rather surprising finding was the drop in serum inorganic phosphate. This decline was small, about 0.5 mg per 100 cc, but was statistically significant. It occurred in all experiments, and the *t* value¹¹ for statistical significance of the difference between the average initial level and that at the middle of the injection was 2.9, a value above the 2.5 required to make it improbable that the decline was fortuitous. The maximal decline occurred at the midpoint at the same time that the amino acids were most rapidly leaving the blood stream.

Since Parenamine is furnished as an unneutralized mixture of amino acids having a pH of about 4.2, it is important to de-

termine whether injections of this product produce any appreciable changes in CO₂ combining power. The results summarized in Table I indicate that the fall in CO₂ combining power is negligible. The range of maximal change was from +3 to -11 cc per 100 cc with an average fall of 4 cc per 100 cc. In 8 out of 13 cases, the fall occurred at the end of the injection. A return to the initial levels had occurred at 2 hours after the completion of the injection.

In 30 experiments listed in Table I, vomiting occurred in 2 cases at or near the end of the injection. In both cases the amino acid N levels were over 11 mg per 100 cc. Two other patients complained of nausea at a time when the amino acid levels were over 10 mg per 100 cc. In all 4 cases the rate of injection was somewhat faster than the average of 3 hours for the 1000 cc. In 5 additional cases not listed, injections were purposely made at fast rates ranging from 90 to 120 minutes for the 1000 cc. In 2 of these experiments, the maximal amino acid N concentration was over 12 mg per 100 cc and were associated with a sense of fullness, nausea, and vomiting. Thus it appears that if the plasma amino acid N concentration rises considerably above 10 mg per 100 cc, there may be symptoms produced comparable to those in acute overeating. It may be that the increase in concentration of a certain amino acid, such as glutamic or aspartic acid, is actually responsible for the symptoms.

¹¹ Snedecor, G. W., *Statistical Methods Applied to Experiments in Agriculture and Biology*, Colleague Press, Inc., Ames, Iowa, 1937.

Amino acid tolerance curves obtained after oral ingestion of 45 g of Parenamine (in another study from this laboratory¹²) were similar to those after intravenous injection except that the average maximal rise was only 3.4 mg per 100 cc, the amino acid N level seldom rising above 10 mg per 100 cc.

Since glucose is frequently administered intravenously along with amino acids, it was found desirable to study the blood chemical changes when a liter of 4.5% solution was injected following the intravenous administration of a liter of 5% glucose. There was no appreciable effect upon the various curves. One such experiment is shown in Fig. 1. The glucose curve fell promptly to normal following the injection. The serum phosphate level, depressed by the glucose injection was

further lowered by the amino acid injection, reaching in one case the low level of 1.5 mg per 100 cc. The rise to normal levels was rapid.

Summary Injection of 45 g of Parenamine intravenously at slow speeds produced an average plasma amino acid N rise of +4.9 mg per 100 cc during the injection with a return to normal in 2 hours. Rises to levels above 10 mg per 100 cc produced by rapid injection may be associated with nausea and vomiting. A progressive though slight rise in blood urea takes place during and after the injection, accompanied by increased urea excretion. Serum inorganic phosphate is lowered during the injection and returns to normal by the end of the injection. Only a negligible drop in CO₂ combining power occurred during the injection. Intravenously injected glucose did not alter the curves produced by the amino acid injection.

¹² Hoffman, William S., and Dyniewicz, H., *Gastroenterology*, in press.

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Is Normal Human Urine Toxic?

BERNHARD ZONDEK, AND RIVKA BLACK

From the Gynecologic Obstetric Department, Rothschild Hadassah University Hospital, and the Hormone Research Laboratory, Hebrew University, Jerusalem, Palestine

Much investigation has been devoted to the question of whether normal urine is toxic. It has been suggested that the increase of a toxic urine constituent is associated with disturbance of renal function (e.g. uremia, eclampsia). Aschheim and Zondek, however, in developing the hormonal pregnancy test concluded that human urine is non-toxic for the mouse, since injections of very large doses of human urine are tolerated by this animal.

Rachmilewitz¹ on the basis of experiments with fibroblast cultures *in vitro* concluded that human urine, native as well as dialyzed,

contains, even in a dilution of 1:50, a powerful toxic substance which inhibits the growth and brings about the degeneration of cells. Uremic and normal urine were found to contain this toxic substance in about equal amounts.¹ It is, however, uncertain whether results of experiments carried out *in vitro* can be applied also *in vivo*, especially where toxicity is in question, since a cell (fibroblast) *in vitro* may lack detoxication potentialities which characterize the intact organism. Many substances whose toxic effect *in vitro* is notorious (e.g. para-chloro-xylenol² and many others) are completely non-toxic *in vivo*. Although dialyzed human urine inhibits the growth of fibroblasts *in vitro*¹ we found that it has no toxic action on a surviving frog

* Partially aided by a grant from the Ella Sachs Plotz Foundation.

¹ Rachmilewitz, M., *Proc. Soc. Exp. Biol. and Med.*, 1940, **43**, 497; *Arch. Int. Med.*, 1941, **67**, 1132.

² Zondek, B., *Nature*, 1942, **149**, 334; *J. Urol.*, 1942, **78**, 747.

TABLE I
Amounts of Normal Human Urine Tolerated Without Toxic Reaction by Different Animal Species

Animal	Wt	cc of urine and distribution of dose	Route of injection	Urine body wt
mice	75 g	6 X 0.4 = 2.4	s c	$\frac{2.4}{75} = \frac{1}{3}$
"	10 "	30 (as 3 cc of dialyzed and conc urine)	"	$\frac{30}{10} = 3$
rats	30 "	90 X 1 = 90	"	$\frac{90}{30} = 3$
"	100 "	30 X 10 = 300	"	$\frac{300}{100} = 3$
rabbit	1500 "	9 X 10 = 90	s c	
"	1000 "	250 (as 20 cc of dialyzed and conc urine)	"	$\frac{250}{1000} = \frac{1}{4}$
dog	15 kg	160	"	

heart (Straub preparation) Native human urine added to the surviving frog heart caused immediate cessation of the heart beat (hyper-tonic salt content) Urine was dialyzed and after 5-fold concentration, rendered isotonic with NaCl This preparation was entirely without toxic effect on the surviving heart The experiment shows that though dialyzed urine may contain a constituent which is *in vitro* toxic to fibroblasts, such urine even in 5-fold concentration *in vivo* is non-toxic not only in respect to the intact organism but even in respect to a surviving organ

In further experiments toleration of normal urine by different species was studied

A dog weighing 15 kg received intravenously 160 cc of native human urine with no undesirable by-effect

An adult rabbit received intravenous injections of 90 cc of native urine in 9 doses of 10 cc each given at intervals of 1 hour The animal showed no toxic response Rabbits weighing 1 kg were injected intravenously with 20 cc of dialyzed urine concentrates, the equivalents of 100-250 cc of native urine These injections elicited no toxic after-effect

Infantile rats (30 g) were given daily in-

jections of 1 cc of native human urine for 3 months, *ie*, a total of 90 cc The onset of sexual maturity was not affected by this protracted treatment with urine Rats weighing 100 g received daily an injection of 10 cc of native human urine for 1 month, a total of 300 cc of urine Nevertheless, a pregnant animal in this group produced a normal litter

Urines were dialyzed and concentrated up to 10-fold *in vacuo* Three cc samples of the concentrates were injected into mice weighing 10 g in 3 injections during 8 hours The mice showed no toxic reaction to these injections, the equivalent of up to 3 times the body weight

Our experiments show that animals tolerate normal human urine about as well as they do physiological saline (Table I)

Urine has been administered for therapeutic purposes to man in recent years both intramuscularly, rectally and orally Ovarian dysfunction has been treated by intramuscular injections of native pregnancy urine, which is rich in gonadotropic and estrogenic hormone In these cases, daily injections of 10-20 cc of pregnancy urine over a period of several weeks elicited no toxic effect Rus-

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¹² Hoffman, William S. and Dymowicz, H., *Gastroenterology*, in press.

15253

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BERNHARD ZONDEK, AND RIVKA BLACK

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contains, even in a dilution of 1:50, a powerful toxic substance which inhibits the growth and brings about the degeneration of cells. Uremic and normal urine were found to contain this toxic substance in about equal amounts.¹ It is, however, uncertain whether results of experiments carried out *in vitro* can be applied also *in vivo*, especially where toxicity is in question, since a cell (fibroblast) *in vitro* may lack detoxication potentialities which characterize the intact organism. Many substances whose toxic effect *in vitro* is notorious (e.g. para-chloro-xylenol² and many others) are completely non-toxic *in vivo*. Although dialyzed human urine inhibits the growth of fibroblasts *in vitro*¹ we found that it has no toxic action on a surviving frog

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¹ Rachmilewitz, M., *Proc. Soc. Exp. Biol. and Med.*, 1940, **43**, 497, *Arch. Int. Med.*, 1941, **67**, 1132.

² Zondek, B., *Nature*, 1942, **149**, 334, *J. Urol.*, 1942, **78**, 747.

A Chick Embryo Technic for Intravenous and Chemotherapeutic Studies *

HENRY F LEE, ABRAM B STAVITSKY, AND MARGARET P LEE
(Introduced by Werner Henle)

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and the Children's Hospital of Philadelphia*

The chick embryo has become increasingly valuable in investigative work and in the production of certain biologic materials. Up to the present time no less than 20 viruses have been propagated by its use. Many of the bacteria, certain rickettsias, leptospira, icterohemorrhagica, and a growing number of types of tissue cells, both benign and malignant, have been studied in the embryo or upon its membranes. Several recent studies have dealt with chemotherapeutic testing as applied to experimentally infected embryos. None of these papers has given detailed quantitative information concerning levels attained by therapeutic agents in the blood and extraembryonic fluids.¹⁻⁵ In the course of the above types of studies many technics have been devised.

Rous and Murphy were the first to use the chorio-allantoic membrane for the study of transplanted tumor fragments. They were also the first to use the chick embryo in the study of an infectious agent, the virus of the Rous Sarcoma.⁶

The majority of studies involving the exposed chorio-allantoic membrane as a site of inoculation have made use of a method of opening eggs first described by Clark.⁷

Woodruff and Goodpasture modified Clark's technic.⁸ Later Goodpasture and Buddingh described improvements and their technic, with slight variations, has been used by many laboratories.⁹ The technic described by Burnet involving the production of an artificial air sac has also found wide use for inoculation of the chorio-allantoic membrane and for infection by the amniotic route.¹⁰ Taylor and Chialvo reported a simplification of Burnet's method which they found particularly valuable for intraamniotic injections.¹¹ A method for intravenous inoculation of the embryo was described by Eichorn.¹² Many modifications of the above technics have been devised.

Each of the technics mentioned is satisfactory for certain purposes. However in the course of experiments concerned with the chemotherapy of bacterial infections produced in chick embryos by intravenous inoculation,[†] it became apparent that no method previously described was entirely satisfactory. A method was required that would permit intravenous inoculation of the embryo as well as accurate injection into or aspiration from any or all of the extraembryonic sacs at the same or at separate times. It was also necessary that the method permit later sampling of the blood. The method here reported permits all of the above procedures as well as placement of

* These studies were supported by a grant from the Heyden Chemical Company.

¹ Bang, F. B., and Bang, B., *Proc Soc Exp Biol and Med*, 1941, **40**, 527.

² Morrow, G., and Berry, G. P., *J Bact*, 1938, **36**, 280.

³ Ransmeier, J. C., *J Infect Dis*, 1943, **72**, 77.

⁴ Weil, A. J., and Gall, L. S., *J Infect Dis*, 1941, **60**, 97.

⁵ Parker, R. F., and Diefendorf, H. W., *Proc Soc Exp Biol and Med*, 1944, **57**, 351.

⁶ Rous, P., and Murphy, J. B., *J A M A*, 1911, **56**, 74.

⁷ Clark, E. R., *Science*, 1920, **51**, 371.

⁸ Woodruff, A. M., and Goodpasture, E. W., *Am J Path*, 1931, **7**, 209.

⁹ Goodpasture, E. W., and Buddingh, G. J., *Am J Hyg*, 1935, **21**, 319.

¹⁰ Burnet, F. M., *Brit J Exp Path*, 1940, **21**, 147.

¹¹ Taylor, R. M., and Chialvo, R. J., *Proc Soc Exp Biol and Med*, 1942, **51**, 328.

¹² Eichorn, E. A., *Science*, 1940, **92**, 245.

[†] To be reported separately.

sian authors have recommended treatment of severe uterine bleeding during puberty by a rectal drip infusion of several liters of native pregnancy urine. The experience of our clinic has corroborated the non-toxicity of this treatment. It may be mentioned also that Atcheson,³ in order to save penicillin, administered urine of penicillin-treated patients to cases of chronic gonorrhea via a duodenal tube with good results.

The conclusion follows from the above findings that normal human urine is non-toxic *in vivo* for animals or man.[†]

Does Ether "Detoxicate" Urine? At the time of development of the pregnancy test, it was found that 7% of urine samples were unsuitable for examination by the method proposed at that time, because their injection into the mouse caused death, mostly within 24 hours. Zondek⁴ reported that "toxic" urines of this type become innocuous to mice if they are first shaken with ether. This observation has since been confirmed by numerous workers. Experiments which are reported below show that the ether treatment is effective not because it extracts a specific toxic constituent but because it frees the urine from microorganisms which contaminate it during the passage over the vulva.

1 An ether extract of "toxic" urine was taken up in oil and injected into mice. It proved completely non-toxic.

Chemical restitution of the extracted urine

³ Atcheson, D. W., *Military Surgeon*, 1944, 95, 58.

[†] Urine may, of course, contain toxic materials, either drugs or hormones, after medication, but such urines cannot be looked upon as normal urines.

⁴ Zondek, B., *Klin. Wschr.*, 1930, 9, 964.

by the re-addition of the ether extract failed to reconstitute toxicity.

Addition of the ether extract to non-toxic urine from another source did not render the latter toxic.

2 Ether had a bacteriostatic or a bactericidal effect on the bacterial flora of the urine. When ether-treated urine which had been allowed to stand at 37° C for 24 hours remained sterile, it proved also to be non-toxic. On the other hand, when development of bacteria occurred in the extracted urine sample during the period of the incubation, the original toxicity of the urine was found to have reverted. *Proteus* bacteria could be isolated from the heart blood of mice which had died following an injection of a "toxic" urine sample. Injection into the mouse of 1 cc of a broth culture of the isolated *Proteus* strain caused death.

3 In all cases, "toxic" urine could be rendered innocuous to the mouse by boiling.

4 Urine was taken by means of a catheter from patients whose urine had proved toxic when it had been taken in the ordinary way. It proved to be non-toxic when it was taken by catheter.

Conclusion. Tolerance tests for urine on a surviving organ (frog heart), and on different species of laboratory animals as well as clinical findings in man prove that human urine is not toxic. The delayed fatal effects which follow the injection of certain urine specimens (7%) into animals are due to infections. Ether treatment renders such urine specimens innocuous not because it removes a toxic substance from the urine but because it is bacteriostatic or bactericidal.

Our thanks are due to Dr. Daniela Weber for bacteriological tests.

TABLE III

Survivals After Opening Shell, Exposing Chorio allantoic Membrane and Sealing with Scotch Tape for the Remainder of Incubation Three Separate Groups of Eggs

Days age	10	15	17	20
Untouched controls	18	18	18	18
Opened group 1	18	18	18	18
" " 2	18	18	16	16
" " 3	18	18	18	18

TABLE III A

Same as Table III Plus Injection of 1 cc of Saline Into the Yolk, Into the Amniotic Fluid, and Into the Allantoic Fluid

Days age	10	15	17	20
Untouched controls	18	18	18	18
Opened No injection	18	17	16	16
" Injected	18	16	15	15

TABLE IV

Intravenous Injection

Survival Rates with .05 cc Saline as Inoculum Injection Time Approximately 5 Seconds
All Control Groups Opened, Membrane Exposed, Sealed

	Age in Days					
	10	11	12	15	18	20
Controls	18	18	18	18	18	18
Large vessels, no hemostasis	48	21	21	18	17	17
Controls	18		16	16	16	16
Fine vessels selected	18		12	11	9	9
Controls	18		18	18	18	18
Fine vessels selected	18		15	14	13	13
Controls		18	18	18	18	18
Fine vessels selected		18		15	15	15
Controls		18		16	16	16
Fine vessels selected		18		14	12	12
Vessel entered with needle but no injection		18		15	14	14

of the membrane. The collection of blood is difficult before the ninth day of incubation.

The open end of the egg is then sealed with 3 layers of transparent Scotch tape. The triple layer is used since it permits the closest approximation to normal water-loss during the remainder of the incubation period as determined by loss of weight (Table I). Substances may later be applied to the chorio-allantoic membrane by simply passing a fine needle through the Scotch tape. At any time the tape may be partially or completely removed for further work. The tape should be applied in such lengths that it extends down over the egg for approximately $\frac{1}{2}$ inch.

Intravenous Injection. Embryos show individual variations of the pattern of veins in the exposed chorio-allantoic membrane. Usually there are several venous branches available for injection. In an occasional egg

no suitable vessels are present in this area. The usual situation is to find one large allantoic vein with two or more tributaries. Very small vessels may be entered with a No. 27 needle provided its point is perfectly sharp and its shaft smooth. It is preferable to use the tributaries and not the main vessel since less bleeding results upon withdrawing the needle. When a large vessel is used it is possible to control the bleeding by the use of very small "serrefine" hemostats. The open jaws of these small spring-activated hemostats are slipped onto the vessel just as the needle is withdrawn and the hemostat is held in place for 15-20 seconds before removal. Hemostasis is complete. An increase in mortality results from this clamping procedure but this increase is not regularly predictable. This step is not necessary if the small vessels are chosen for injection.

TABLE I
Average Water Loss (g) 10th Through 20th Day of Incubation Each Figure Is
Average of 12 Eggs

Wt loss in g	Unopened eggs	Opened, membrane exposed, single layer Scotch tape	Ditto double layer	Ditto triple layer
	40	77	51	46

TABLE II
Volume of Blood (cc) Obtained from Embryos of Various Ages

Embryo No	Age in Days						
	10	11	12	13	15	17	18
1	08	20	23	26	30	39	70
2	05	18		29	32	42	70
3	10	23		31	33	40	50
4	09	20		31	29	33	60
5	12	28		20	36	43	65
6	12	10	24	34	36	43	75
Avg	09	19		28	32	39	65

material upon the exposed chorio-allantoic membrane

Technic Eggs of the selected incubation-age are candled and the air sac margin is marked with a pencil. For intravenous work the embryo should be of at least 10 days incubation. The shell along the air sac margin is wiped off with 70% alcohol. When many eggs are handled a squat, wide-mouthed bottle filled with 70% alcohol with a pad of cotton over its mouth facilitates this step. The cotton is allowed to project into the alcohol-bearing cotton. The shell over the air sac is removed by cutting just distal to the pencil line with a thin carborundum disc rotating at high speed. The motor is fixed in a vise and the egg rotated in contact with the cutting disc. With a little practice this "decapping" procedure can be done very rapidly. It is possible to remove the shell cap entirely or to leave it attached by partially cut shell so that it serves temporarily as a cover against air-borne dust. If the cap has been left attached it is easily removed with cover-slip forceps. The egg is inverted and tapped lightly to remove shell dust from the surface of the reflected portion of the shell membrane. The rest of the work requires a satisfactory egg-holder. A low wide-mouthed bottle with its open end covered with cotton moistened with 70% alcohol is

ideal for this purpose. The cotton supports the egg firmly but permits instant adjustment of position. Good lighting is also important. A light source in front of the operator has been found best, provided that a piece of Polaroid is placed between the light source and the egg. The Polaroid serves to eliminate glare from the moist membrane surfaces.

As recommended by Burnet in his description of a side-window technic about 0.1 cc of sterile saline solution is placed over a slight nick made in the shell membrane overlying the chorio-allantoic membrane. The nick is made with the fine needle which deposits the saline. The saline solution serves to initiate separation of the shell membrane from the underlying chorio-allantoic membrane. In the presence of this saline the shell membrane may then be gently picked up with fine-pointed forceps and stripped off with no resultant capillary bleeding. The entire surface of the chorio-allantoic membrane lying under the air sac may be exposed or as much of this area as is desired for the particular work being done. Injection intravenously, into the amniotic fluid, into the allantoic fluid, or into the yolk sac, may then be made under full visibility. Blood or extra-embryonic fluids may be collected or materials may be deposited upon the surface

Blood Levels Attained with Compounds Administered by the
MEMBRANE SURFACE ROUTE

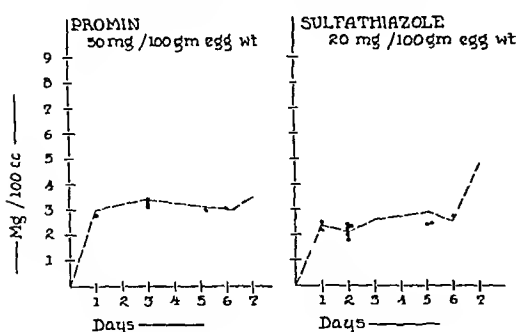


Fig 1

up and torn in this area. The allantoic vein is grasped with forceps and a No 25 needle is inserted. The needle is advanced into the vein for approximately one-quarter inch. The forceps may then be removed and blood gently aspirated. The amount of blood obtainable from the embryos of various ages is shown in Table II.

Chick embryos are notably resistant to accidental infection, and it has been the experience of others that it is possible to work in an open room without any special precaution against air-borne contamination. The studies here recorded were carried out as aseptically as possible under a hood. Occasional contamination occurs under the best of conditions.

Advantages of the Technic The principal advantages of the technic may be summarized as follows:

1 Opening and sealing of eggs is relatively rapid.

2 Adequate exposure is obtained for selective or combined intravenous, intra-yolk, intra-amniotic or intra-allantoic injection or aspiration.

3 All structures are clearly visible, thereby assuring accuracy of placement of injected materials.

4 The same egg may be used for later bleeding and/or sampling of the extra-embryonic fluids.

5 Embryo and membranes remain visible during incubation. Candling is unnecessary since death of the embryo is immediately

apparent through the large transparent window.

6 The feasibility of selecting very small allantoic venous tributaries for intravenous injection minimizes bleeding.

Mortality from Procedure When using embryos of 10 days incubation or more the mortality resulting from opening the shell and exposing the chorio-allantoic membrane is negligible (Table III). Injection of sterile saline solution into the amniotic, allantoic, or yolk sac does not increase the mortality (Table III, A).

Following intravenous injection of sterile saline solution the mortality rises sharply when using 10-day embryos. With the use of 11-day embryos the survival rate improves somewhat (Table IV). Volume of injection, within limits, is more important than rate of injection (Table V). In addition to the data given in these tables, a series of experiments involving controls receiving intravenous saline solution has demonstrated a uniformly higher survival rate when 11-day-old embryos are used as compared to 10-day-old embryos. This age factor is important principally in regard to intravenous work. For other types of work, survivals are excellent with embryos 5 days of age and up.

Quantitative Studies of Compounds Administered to Chick Embryos Although the chick embryo is being used for pilot testing of compounds for possible chemotherapeutic value, there are few data available concerning the distribution of such compounds after their placement within the egg. It has been shown that certain sulfonamides are rapidly distributed through the egg but reports are chiefly qualitative.^{2,3,4} Depending upon the route of inoculation and upon the conditions of a given experiment, information concerning levels attained in yolk, amniotic, and allantoic fluid may be of value. Experimental infection of the embryo itself may be produced by intravenous inoculation. Under such circumstances a knowledge of blood levels reached by chemotherapeutic compounds makes possible a more definitive interpretation of results.

Two compounds were chosen for quantitative studies. One, sulfathiazole (2-(p-amino-

TABLE V
Intravenous Injection of Saline Effect of Volume and Rate of Injection

Days old	10	11	12	13	14	15	16	17	18	19	20
A Effect of Rate of Injection											
Controls	18			16			16			16	16
05 cc in 2 sec approx	18			9			9			9	9
05 cc in 5 sec approx	18			12			11			9	9
05 cc in 60 sec approx	18			11			11			8	8
B Effect of Volume of Injection											
Controls	18			18			18			18	
05 cc in 5 sec approx	18			15			14			13	
1 cc in 5 sec approx	18			9			4			3	
2 cc in 5 sec approx	18			3			1			1	

TABLE VI

Compound	No of embryos	Dose in mg per 100 g of egg wt	% survival* after 9 days
Promin	18	60	0
	18	50	50
	18	40	80
	18	30	100
	18	20	50
Sulfathiazole	18	15	84
	18	10	100
	12	5	0
	12	3	75
Promizole	12	2	92
	12	1	100
	12	5	0
	12	2	75
3,3' dimethyl 4,4' diaminodiphenyl sulfone	12	1	100
	12	5	0
	12	2	59
	12	1	81
3 methyl 4,4' diaminodiphenyl sulfone	12	5	100
	12	5	0
	12	2.5	0
	12	1.5	46
	12	1	83
	12	5	92

In all cases above compounds were injected into yolk sac on the 10th day of incubation. Regardless of route of administration it was found that comparative toxicity remained the same.

* All figures are corrected for mortality of controls

Quarter cc tuberculin syringes and $\frac{1}{2}$ inch No. 27 needles have been found most suitable ‡

Collection of Blood Samples Bleeding is accomplished by one of two methods. If it is desired to obtain more than one sample from an embryo one of the branches of the allantoic vein must be used. A citrated

1.5 cc syringe and a No. 25 needle is suitable. When withdrawing blood after entering the vein, very gentle intermittent traction on the syringe plunger is employed. When it is desirable to obtain as large a blood specimen as possible, it is best to use the large main trunk of the allantoic vein as it proceeds toward the embryo in a fold of membrane. An avascular area of the chorio-allantoic membrane is selected. With two pairs of fine pointed forceps the membrane is picked

‡ Results obtained by the use of this method for intravenous inoculation with suspensions of tubercle bacilli will be the subject of a separate report.

Relationship of Quantitative Levels Attained by Compounds when Administered by MEMBRANE SURFACE

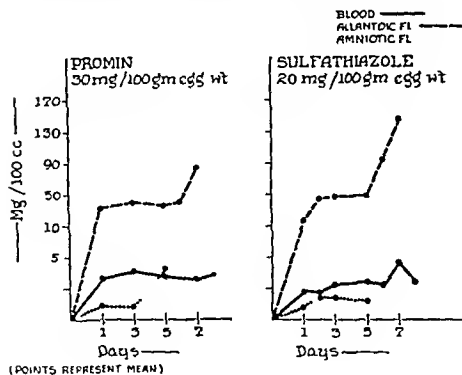


Fig 5

Relationship of Quantitative Levels Attained by Compounds when Administered by the YOLK SAC ROUTE

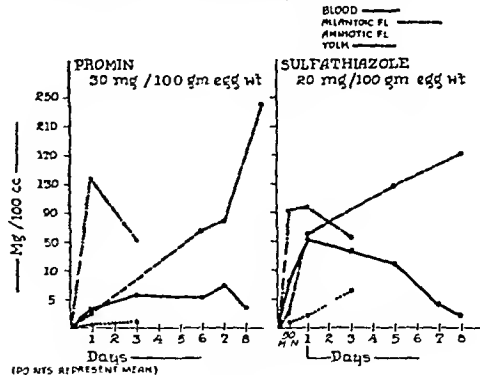


Fig 6

other fluids were done and suffice to give an indication of the distribution of the compounds. A total of 850 quantitative determinations were made. Studies with low doses and with toxic doses are omitted from the tables. Determinations were made by the Proom modification of the Marshall method¹³.

In all cases the figures represent single specimens from individual embryos and not serial specimens from the same embryo. It is possible, however, when such data is needed, to obtain at least 3 blood specimens at intervals from an individual embryo.

The few streptomycin determinations that have been completed indicate that this substance is present in the blood in a concentration of 6 to 10 units per cc of blood 12 hours after the placement of 1000 units on the surface of the chorio-allantoic membrane. Higher levels over longer periods are maintained in the allantoic fluid.

Discussion Despite its limitations, which must be clearly recognized, the chick embryo is a valuable laboratory aid for screening procedures in chemotherapeutic research. It presents both simple (membrane mesenchyme)

and highly complex tissues for study. Because of its immunologic status it is adaptable to certain bacteriologic problems wherein tissues are needed which are, so far as has been ascertained, free from antibody and sensitization phenomena. Within the egg one may study infection in the presence of living parenchymatous organs with their enzyme systems and metabolic processes intact. Naturally occurring infection of any sort in the chick embryo is a rare occurrence. A large number of embryos may be maintained in scarcely more space than is required for tissue culture studies. It is believed that the technic here presented may serve to facilitate such investigations as may make use of the chick embryo.

Summary A chick embryo technic is described which possesses certain advantages over reported methods. The technic is applicable to many types of investigation. Quantitative data are presented on the distribution of sulfathiazole and promin within the fertile egg.[§]

[§] This work was carried out in laboratory space provided by The Henry Phipps Institute of the University of Pennsylvania.

¹³ Proom, H, *Lancet*, 1938, 1, 260

Blood Levels Attained with Compounds Administered by the
YOLK SAC ROUTE

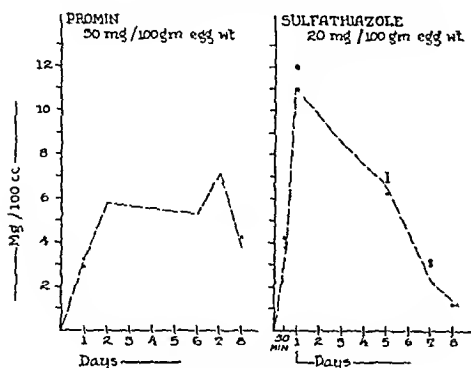


Fig 2

Blood Levels Attained with Compounds Administered by the
AMNIOTIC FLUID ROUTE

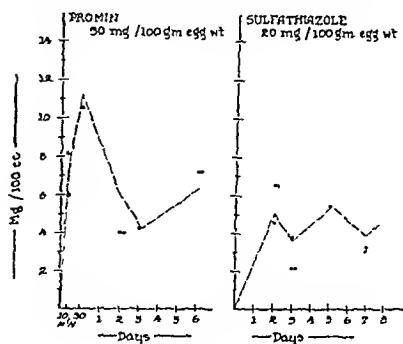


Fig 3

Blood Levels Attained with Compounds Administered by the
ALLANTOIC FLUID ROUTE

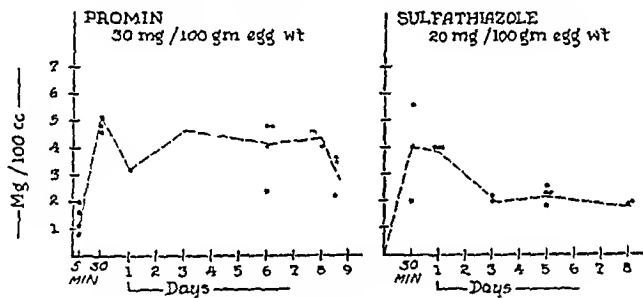


Fig 4

benzenesulphonamido)thiazole), is a relatively insoluble sulfonamide while the other, Promin, (sodium p-p' diaminodiphenylsulfone N-N' didextrose sulfonate), is a highly soluble sulfone. In addition a few streptomycin assays have been completed.

Methods The technic described above was used throughout. The compounds were administered as a 5% solution in the case of Promin or as a 5% suspension of micro-crystals in the case of sulfathiazole. Dosage was computed in terms of milligrams per 100 g of egg weight. Although the weight of the eggs used varied from 48 to 64 g, the dosage was computed on the basis of the uniform egg weight of 55 g. This introduces an error in dosage not exceeding 15%. All possible routes of administration were tested.

Toxicity studies were carried out first. Maximum tolerated doses by the yolk sac route were determined. The findings for

promin, sulfathiazole, and 4 other compounds are given in Table VI. For these toxicity studies the compounds were injected into the yolk sac on the 10th day of incubation. Toxicity was determined in terms of embryo survival to the 20th day of incubation. The figures are corrected for the mortality of controls from the same group of eggs. The controls were handled exactly as the test embryos except that no material was injected. Eighteen embryos were used for each dosage group for the promin and sulfathiazole, while in the case of the other compounds 12 were used for each group.

On the basis of these toxicity studies dosages of the 2 compounds were selected for quantitative studies. The following graphs reveal the results obtained. All figures represent milligrams per 100 cc of fluid or blood. Although the emphasis was placed upon blood levels, a number of determinations in the

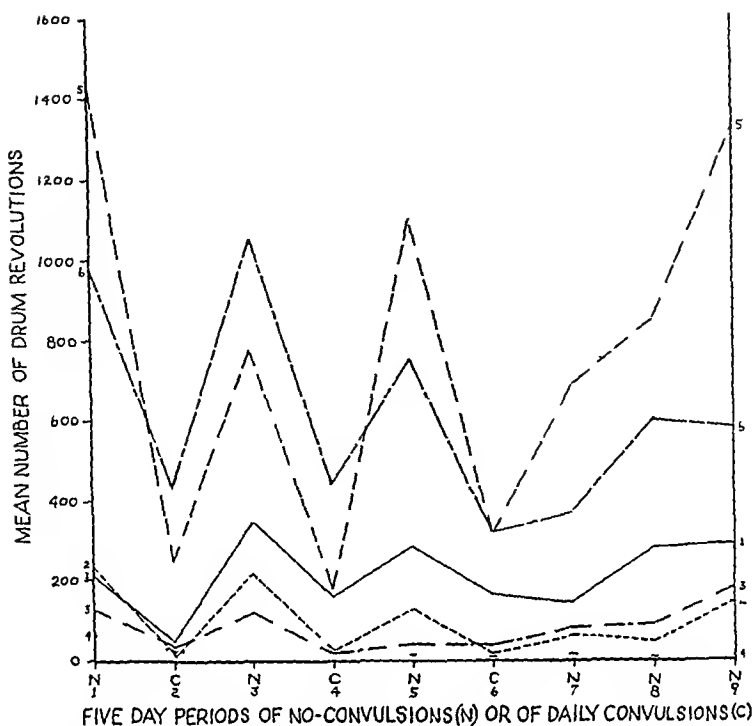


Fig 1

Activity records of 6 female rats. In periods 1, 3, 5, 7, 8, and 9 the usual conditions for rats in activity drums prevailed, whereas, in periods 2, 4, and 6 the rats received one electro convulsive shock each day. Each rat's number is given on the left and right ordinate.

sive shock. Eventually, however, a disturbance of estrus might have been a contributing factor. Further studies on this and other aspects of the problem are in progress.

Conclusion Electro-convulsive shocks in 6 female rats greatly reduced the amount of their voluntary activity for a short period

of time. The major change appears within the first 24 hours and disappears within 48 hours after the last convulsive shock is administered. Within a 2-week period following their last convulsion the majority of the animals closely approximated their pre-shock levels of daily activity.

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Changes in the Hydrogen Ion Concentration of Healing Fractures

ORVAR SWENSON AND C LLOYD CLAFF (Introduced by E C Cutler)

From the Laboratory for Surgical Research, Harvard Medical School, Boston, Mass

The repair process involved in fracture healing is of great interest both from theoretical and practical standpoints. Repair of bone differs from soft tissue repair, in

that the process involves calcification and ossification. The morphological changes in fracture repair are well established and have recently been reviewed by Urst and Mc-

Effects of Electro-Convulsive Shocks on Daily Activity of Albino Rats in Revolving Drums

CALVIN P. STONE

From the Department of Psychology, Stanford University

Exploratory activity and rate of locomotion of rats in mazes and other learning situations falls off sharply if they are subjected to a series of electro-convulsive shocks^{1,2}. Their performances are characterized by delays in starting, frequent pauses, and lethargic movements. These observations suggest the desirability of further study of the voluntary activity of rats in non-learning situations such, for example, as the Slonaker-type of revolving drums. A preliminary study was undertaken with 6 adult females.

For the first 3 weeks no convulsive shocks were administered. During this time the animals had established characteristic trends of daily activity which, although variable from day to day, nevertheless seemed sufficiently stable to enable one to detect sudden deviations of large magnitude. At the beginning of the fourth week each rat received a convulsive shock daily for 5 days. This was followed by 5 days without shock, 5 days with daily shock, and so on, for 3 alternating periods of shock and no-shock, plus 2 additional post-shock periods.

To cause the convulsion, a 70-volt alternating current was applied to the rat's scalp over the forebrain for approximately 2 second through alligator-clip electrodes. The electrodes were fastened on the dorsal scalp approximately 6 mm apart and 1 cm behind an imaginary line between the eyes. The scalp was moistened with a salt solution where the clips were attached. Each convulsion had the characteristic features of a grand mal seizure, starting with vigorous tonic contractions and passing into the clonic phase after about 10 seconds. The total seizure lasted between 20 and 30 seconds. Immediately after the convulsion was over most

of the animals were hyposensitive to mild external stimuli for from 2 to 3 minutes. Then followed a short period of hypersensitivity, ushered in by startle reactions or mulling about the cage when lightly stimulated by the experimenter or a cage mate. Occasionally during the hypersensitive stage an animal would attempt to bite the hand of the experimenter. The animals were replaced in the activity drum immediately after their shocks where they were free to sleep or to rotate the drum. Only one animal (No 1) regularly revolved the drum soon after the convulsion, this occurring while she was in the hypersensitive stage.

In Fig 1 are given the mean numbers of revolutions made by each animal during nine successive 5-day periods. The characteristic level of each rat's activity just prior to the first series of convulsions is shown in period 1. In periods 2 to 7 appear the means for alternating periods of shock and no-shock. Periods 8 and 9 show the gradual return to the pre-shock level of activity on the part of most of the rats.

As is apparent, the rats were much less active during the shock periods than during the no-shock periods. Reduction began in the first 24 hours after a convulsive shock and disappeared during the second day beyond the last shock. Rat No 4, a relatively inactive animal, was an exception to this generalization, however. It ceased running almost altogether after its first period of electro-shocks and did not again become active during the 2 weeks beyond the last convulsion.

In this preliminary study we did not attempt to correlate changes in activity with possible disturbances of the estrus cycles. Nevertheless, the suddenness with which activity was depressed by the first convulsion clearly suggests that the primary reduction was due to some direct effect of the convul-

¹ Stainbrook, E. J., *J. Psychol.*, 1942, **13**, 337.

² Siegel, P. S., *J. Compar. Psychol.*, 1943, **36**, 61.

tions of the wall near the beveled end. Only rarely did infection of the fracture follow. Most infections seemed to be caused by contamination at operation as the signs of infection appeared early. Late infections attributable to repeated aspirations were rare. Many of the fractures made were unsuitable for study, as the amount of hematoma fluid was insufficient to yield fluid by repeated aspirations for the 7 to 15 days necessary for calcification, demonstrable in a biopsy of the fracture site. Small hematoma and infection made many of the fractures produced unsuitable for study.

Aspiration of the hematoma was carried out $2\frac{1}{2}$ hours after the fracture was produced, again 24 hours later, then every 48 hours until fluid was unobtainable. pH determinations were made in duplicate by means of a micro-glass electrode, designed by one of us—(C. L. C.), capable of making readings on as little as 0.008 ml of fluid. Biopsies were taken at various intervals to correlate the morphology with the pH changes, and to check specifically for beginning calcification by the Kossa staining technic.

Results The first aspirations, performed $2\frac{1}{2}$ hours after the fracture was produced, yielded a bloody fluid with a pH more acid than that of the venous blood. In a series of 8 fractures, ($2\frac{1}{2}$ hours after the fracture was produced) the average pH of the fluid was 7.30, and 24 hours later, the pH in the 8 fractures averaged 7.18. No analysis was carried out to try to determine the substances responsible for the local acidity.

A fracture becomes the center of an intense acute non-infectious inflammatory reaction and, according to Menken,⁸ the pH initially in such a reaction should be definitely acid. Breakdown of tissue, damaged at the time of fracture, with more or less isolation of the site of injury from the general circulation, contributes to the local acidity.

As repeated aspirations were performed, at 48-hour intervals, the fluid gradually changed

to the alkaline side. In the 8 fractures the average pH at the end of the experiment was 7.52. The shift to the alkaline side, as repair takes place, is difficult to explain. Certainly as the site of injury is vascularized, the local acidity is neutralized. However, this does not explain the peculiar local change to the alkaline side.

The end of each of the 8 experiments was determined by inability to aspirate sufficient fluid for pH determinations and varied from 7 to 15 days. Biopsy of the fracture site was carried out at the termination of each experiment and in 6 out of 8 there was calcification demonstrable by the Kossa staining technic. The material from the remaining two experiments showed questionable calcification. Six other fractures were biopsied from 3 to 5 days after fracture when the pH of the hematoma was still on the acid side or about the same as the venous blood and in none of these was it possible to demonstrate calcification.

The rôle of the hydrogen ion concentration in calcification and ossification is difficult to ascertain. A major rôle has been ascribed to it by the exponents of a theory of calcification.^{4,5} This postulates that there is initially a high concentration of calcium ions at the fracture site, due to the increased solubility of the bone calcium salts in the acid hematoma fluid. Actually no measurements of calcium have been reported confirming this theory. As the calcium salts are less soluble in an alkaline solution, it is further postulated that precipitation of calcium takes place as the hematoma at the fracture site becomes alkaline and repair progresses. Certainly such changes in the pH do take place in the hematoma. However, calcification is probably a more complicated mechanism than this theory sets forth. These changes in pH values may be results rather than the controlling factor in calcification.

A persistently low pH at the fracture site invariably indicated infection. It was possible to detect an infected fracture by the persistence of low pH readings before local signs of infection developed. As long as local infection persisted, it was impossible to

⁷ Cluff, C. L., and Swenson, O., *J. Biol. Chem.*, 1944, 152, 519.

⁸ Menken, V., *Am. J. Path.*, 1934, 10, 193.

TABLE I
pH Changes in Healing Fractures

Time after fracture		2½ Hrs	24 Hrs	3 Days	5 Days	7 Days	9 Days	11 Days	13 Days	15 Days	
pH	Hematoma	220 43	7 18	7 07	7 19	7 22	7 29	7 36	7 32	7 47	7 53
"	"	217 43	7 42	7 33	7 26	7 28	7 29	7 51			
"	"	235 43	7 34	7 25	7 37	7 42	7 52				
"	"	215 43	7 28	7 25	7 29	7 24	7 51	7 46			
"	"	309 43	7 35	7 08	7 24	7 31	7 41				
"	"	333 43	7 36	7 36	7 25	7 30	7 41	7 54			
"	"	3 44	7 15	7 13	7 30	7 34	7 44	7 54			
"	"	127 43	7 30	6 90	7 02	7 23	7 42	7 60			

Lean¹²³ Less complete biochemical knowledge of fracture repair is available. Clinicians are interested in the process of calcification and ossification, as they are at a loss at times to explain nonunion of fractures or to alter the clinical course of this complication without extensive surgical procedures.

Detection of any postulated biochemical disturbance in calcification in nonunion presents an almost insurmountable problem. First of all, little is known of the biochemistry of normal fracture repair. Most investigations of fracture healing have been morphological studies. Correlation of the biochemical changes with the morphological changes would be interesting and instructive. Analyses of tissue in the region of healing fractures have been made. However, in the early stages of repair there is only a liquid hematoma at the fracture site and tissue analysis is impossible. The presence of the hematoma is fortunate in one respect in that it is comparatively simple to draw out fluid and subject it to chemical analysis. While the hematoma does not represent accurately the biochemical processes in early fracture repair, it probably reflects these factors sufficiently to yield reliable information concerning changes in concentration of various substances as preparations for calcification take place. Assuming that this is true, aspiration of the hematoma and analysis of the fluid is a relatively simple technic for biochemical study of fracture repair. A major problem is the small volume of hematoma

fluid present at the site of fracture necessitating the use of microchemical methods.

Determination of the hydrogen ion activity of the hematoma at fracture has been made by Murray,⁴ Sterling,⁵ and Greune.⁶ Technical difficulties seriously limited the number of determinations that were made. Murray and Sterling generally agree, both reporting a pH on the acid side initially, with subsequent change to the alkaline side. In several experiments Greune found a tendency of the fracture site to remain on the acid side. No mention is made of any evidence of infection in these fractures. It was our experience in all instances that persistent acidity at the fracture was accompanied by infection. A desire to determine the timing and extent of pH changes during fracture repair prompted these experiments.

Methods The first problem was to produce a fracture with a hematoma of adequate size, which remained fluid sufficiently long to permit repeated aspirations for pH determinations. Numerous trials established that the best method to produce such a fracture in a canine femur was to elevate a full thickness spicule of bone and excise a portion of the vastus lateralis muscle, thus providing room for an ample hematoma. Heparin was used in some hematomas in the hope of maintaining a more prolonged fluid state. This technic was of little avail and heparin was not used in the later experiments.

Aspiration of the hematoma was done with 16 gauge needles, having multiple perfora-

¹ Urist, M. R., and McLean, F. C., *J. Bone and Joint Surg.*, 1941, **23**, 1

² Urist, M. R., and McLean, F. C., *J. Bone and Joint Surg.*, 1941, **23**, 283

³ Urist, M. R., *J. Bone and Joint Surg.*, 1942, **24**, 47

⁴ Murray, C. R., *Indust. Med.*, 1941, **10**, 171

⁵ Sterling, R. I., *Tr. Med. Chir. Soc. Edinburgh*, pp. 203-228, 1931-1932, in *Edinburgh M. J.*, Dec. 1932

⁶ Greune, H., *Deutsche Ztschr. f. Chir.*, 1931, **203**, 324

TABLE I
Development of Blood Dyscrasias in Rats Given Thiourea in Purified Diets With and Without the Simultaneous Administration of Thyroxin or Thyroid Powder

Diet	No of rats	No with granulocytopenia*	No with leucopenia†	No with anemia‡
Basal control	21	0§	0§	0
Containing thiourea (No 985)	28	1	7	14
Basal diet and thyroxin	16	1	0	0
Diet containing thiourea (No 985) and thyroxin	16	10	10	1
Diet containing thiourea and thyroid powder (No 1053)	30	18	20	2

* 300 or fewer polymorphonuclear granulocytes per cu mm

† 4000 or fewer total leucocytes per cu mm

‡ Hematocrit value of 35 volumes % or less

§ White cell counts were obtained on only 9 of the 21 control animals

0.25% of thyroid powder (Armour USP)

The study consisted of 2 parts, (a) a series of experiments designed to study the development of blood dyscrasias under various conditions and (b) a test of the value of *L. casei* factor in correcting granulocytopenia. For each experiment in the first part of the study weanling male albino rats of the Osborne and Mendel strain were divided into 2 (or 4) groups on the basis of weight and litter. One group was given the basal diet and another the experimental diet containing thiourea (No 985), when 4 groups were employed the 2 additional ones received these identical diets and in addition were given thyroxin injections. The thyroxin (crystalline, Squibb) was administered subcutaneously 6 times weekly at a level of 1 µg per gram of body weight. In the second part of the study, diet No 1053, containing thiourea and thyroid powder, was given to all animals throughout the entire experiment. A number of the rats which became granulocytopenic were treated with *L. casei* factor, diet No 1053 being continued.

At various times, total white blood cell counts, polymorphonuclear granulocyte counts and hematocrit determinations were made on the tail blood of these animals by technics which have been described.⁶

For this report, rats with counts of no more than 300 polymorphonuclear granulocytes per cu mm were considered to be granulocytopenic, those with counts of 4000 or fewer total white cells per cu mm were considered to be leucopenic, and those with hematocrit values of 35 volumes % or less were considered to be anemic. The incidence of granulocytopenia, leucopenia and anemia in the animals subjected to the different experimental conditions is given in Table I. The lowest values observed have been used in the preparation of this summary.

Of 28 animals which were given thiourea but no thyroxin or thyroid powder, only 1 became granulocytopenic while 7 became leucopenic and 14, or 50%, became anemic. Eight of these 14 had hematocrit values of 30 volumes % or less. Results at hand suggest that the anemia cannot be prevented by the inclusion of *L. casei* factor or a crude liver fraction in the diet but the data are not sufficiently extensive to permit a definite conclusion. Animals which received thiourea but also received thyroxin injections or thyroid powder in the diet showed a low incidence of anemia (3 of 46) but a high incidence of granulocytopenia (28 of 46) and leucopenia (30 of 46). Control animals which received no thiourea showed almost no blood dyscrasias (1 of 37) whether or not they were given thyroxin injections.

As a rule, the rats developed the blood dyscrasias after only a few weeks on the experimental diets. The anemia usually appeared somewhat earlier than the granulocytopenia. As noted above 8 rats given the

⁶ Dift, P. S., Kornberg, A., Ashburn, L. L., and Sebrell, W. H., *Pub Health Rep*, 1945, 60: 1201.

demonstrate calcification until the inflammation subsided

Conclusions 1 In the dog, there is an initial local acidity in the hematoma resulting from a fracture, followed by a change to alkalinity beyond the value in the venous

blood

2 Biopsy of the fracture site after the fluid became alkaline, at the end of the experiment, showed calcification in 6 out of 8 cases

3 Infection at the site of fracture produced a persistent local acidity

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Granulocytopenia in Rats Given Thiourea and Thyroxin The Therapeutic Effect of *L casei* Factor

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The effect of thiourea and its derivatives in depressing the functional activity of the thyroid gland in experimental animals and in man is well known and the literature pertaining to the discovery of this phenomenon has been repeatedly reviewed^{1,2} In the first report on the clinical use of thiourea and thiouracil in the treatment of thyrotoxicosis¹ there was noted the development of agranulocytosis in one patient A number of additional cases of leucopenia, granulocytopenia or agranulocytosis following the use of these drugs have been described The importance of finding a method for preventing the development of these blood dyscrasias is obvious It appeared that there might be an analogous situation in the occurrence of granulocytopenia in rats fed sulfonamides in purified diets^{3,4} and the correction of this dyscrasia with *L casei* factor⁵ It was decided, therefore, to study the effect on rats of administering thiourea in a similar purified diet

with and without the simultaneous administration of thyroxin or thyroid powder If granulocytopenia developed it would then be possible to test the therapeutic value of *L casei* factor in these animals

Anemia without granulocytopenia has been encountered in many of the animals receiving thiourea without thyroxin or thyroid powder Granulocytopenia without anemia has occurred in high incidence in animals receiving both thiourea and thyroxin or thyroid powder and the granulocytopenic animals have been treated successfully with *L casei* factor

Experimental The basal (control) diet used in these investigations consisted of leached and alcohol-extracted casein 18%, Crisco 8%, modified Osborne and Mendel salt mixture³ 4%, $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ 0.18%, $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ 0.02% and anhydrous dextrose (Merck's U.S.P.) 69.8% Into each 100 g of diet were incorporated 1 mg of thiamine hydrochloride, 2 mg of riboflavin, 2 mg of calcium pantothenate, 1 mg of pyridoxine hydrochloride and 200 mg of choline chloride Each rat received a supplement twice weekly of 0.25 cc of corn oil containing 2000 units of vitamin A and 200 units of vitamin D (Natola) The experimental diets differed only in that specified ingredients were substituted for equivalent amounts of dextrose Diet No 985 contained 1% of thiourea, diet No 1053 contained 1% of thiourea and

¹ Astwood, E B, *J Am Med Assn*, 1943, **122**, 78

² Miner, J P, Reineke, E P, and Turner, C W, *Endocrinology*, 1944, **34**, 168

³ Spicer, S S, Daft, F S, Sebrell, W H, and Ashburn, L L, *Pub Health Rep*, 1942, **57**, 1559

⁴ Kornberg, A, Daft, F S, and Sebrell, W H, *Science*, 1943, **98**, 20

⁵ Daft, F S, and Sebrell, W H, *Pub Health Rep*, 1943, **58**, 1542

marrow studied in paraffin sections showed in many rats striking increase in erythropoietic activity and some decrease in number of granulocytes. The majority of the nucleated red cells were normoblasts. The only abnormal finding in the 13 control rats was lipid depletion of the adrenal glands of 4 rats, slight in 3, moderate in 1.

The adrenal hemorrhage and necrosis are almost completely prevented by thyroxin injections or by thyroid powder in the diet. Of 26 animals examined which had received thiourea, but no thyroxin or thyroid powder, 15 showed hemorrhage or necrosis or both of the adrenal. Of 15 animals which had received thyroxin injections in addition, and of 8 which had received thiourea and thyroid powder, 2 (one in each group) showed similar lesions. It would appear, therefore, that this damage to the adrenal glands is mediated in some way through the thyroid. The lipid depletion is not prevented by the injection of thyroxin or feeding thyroid powder.

Discussion During the course of this work there appeared a report⁸ describing the occurrence of granulocytopenia in rats fed a standard laboratory ration containing 0.5% of thiourea. The animals apparently developed neither leucopenia nor anemia but the average neutrophil percentage dropped from 32.7 to 5.4. It was reported that this granulocytopenia could be prevented by the inclusion of 5% of a crude liver fraction in the diet.

The anemia observed in our rats ingesting thiourea but receiving neither thyroxin nor thyroid powder apparently resulted in some way from the interference of the drug with thyroid function. This is suggested very strongly by the low incidence of anemia in the animals receiving thyroxin or thyroid powder in addition to the thiourea. These findings are in accord with the reported occurrence of anemia in clinical hypothyroidism and in thyroidectomized rats.⁹

It should be noted that the granulocy-

topenia in these experiments has been, comparatively, very difficult to correct. Rats with granulocytopenia induced by other methods have responded spectacularly and with considerable regularity to treatment for shorter periods and with smaller amounts of the vitamin. For example, in a recent study of the treatment of sulfasuxidine-induced granulocytopenia,¹⁰ 11 of 14 animals responded to the administration of 25 μ g of *L casei* factor daily for 4 days. In these 11 animals the level of circulating granulocytes increased in 4 days from an initial average of 10 cells per cu mm to an average of 4530 cells per cu mm. Similar responses to identical therapy were obtained in granulocytopenic animals in which the dyscrasia was induced by diet alone,¹¹ by a deficiency of pantothenic acid,⁶ or by a deficiency of riboflavin.¹² The granulocytopenia appearing in animals ingesting thiourea and receiving thyroxin or thyroid powder may differ therefore from the granulocytopenia resulting from these other conditions. It may be that the effect of the thyroxin and thyroid powder is to increase the animals' requirements for *L casei* factor. A similar effect of thyroxin has been demonstrated for other vitamins of the B-complex.¹³ The effect of the thiourea is conceivably due to a direct interference with some metabolic activity in the animal body.

It is of particular interest that the production of granulocytopenia by thiourea in these studies required the simultaneous administration of thyroxin or thyroid powder, each of which was given at a high level. These conditions are perhaps somewhat similar to those under which clinical agranulocytosis follows the use of thiourea.

Summary Rats given thiourea in a purified diet develop anemia and, in lesser incidence, leucopenia. They also develop hemorrhage

¹⁰ Endicott, K. M., Daft, F. S., and Ott, M., *Arch. Path.*, 1945, **40**, 364.

¹¹ Kornberg, A., Daft, F. S., and Sebrell, W. H., *Proc. Soc. Exp. Biol. and Med.*, 1945, **58**, 46.

¹² Kornberg, A., Daft, F. S., and Sebrell, W. H., *Arch. Biochem.*, 1945, **8**, 431.

¹³ Drill, V. A., and Overman, R., *Am. J. Physiol.*, 1941 **42**, 135, 474.

⁸ Goldsmith, E. D., Gordon, A. S., Finkelstein, G., and Charapier, H. A., *J. Am. Med. Assn.*, 1944, **125**, 847.

⁹ Crafts, R. C., *Endocrinology*, 1941, **29**, 596.

TABLE II
Treatment of 12* Granulocytopenic Rats with *L. casei* Factor (100 μ g Per Rat Per Day)
for 4 and 10 Days

	Total white blood cells per cu mm		Total polymorphonuclear granulocytes per cu mm	
	Avg	Range	Avg	Range
Before treatment	4630	1300 8850	140	50 300
After 4 days	5470	1650 9250	630*	50 1100*
" 10 "	7920	2950 13,800	2030	800 4100

* A total of 14 animals were treated. One died on the 7th day of treatment and one responded sufficiently well in 4 days that treatment was discontinued. For the sake of uniformity the data for these 2 animals are not included in the table. The animal which responded in 4 days did so on 2 occasions, once from 200 up to 6700 and again from 250 up to 1400 polymorphonuclear granulocytes per cu mm.

thiourea-containing diet (No 985) became rather severely anemic (hematocrit values of 30 volumes % or less). This degree of anemia developed in these 8 animals usually after about 1 month on experiment (22 to 33 days for 7 animals, average 26 days, 181 days for the eighth animal). The 28 rats which became granulocytopenic while receiving thiourea plus thyroxin or thyroid powder developed this dyscrasia after 25 to 104 days on experiment (25 to 55 days for 25 animals, average 38 days, 75, 81 and 104 days, respectively, for the other animals).

Granulocytopenic rats fed diet No 1053, containing thiourea and thyroid powder, were used in tests of the therapeutic value of *L. casei* factor*. In preliminary trials it was found that treatment of such animals for 4 days with 25 μ g of *L. casei* factor daily did not routinely correct the dyscrasia. The therapeutic dose was increased to 100 μ g daily but as indicated in Table II the response even to this amount was not spectacular. Only 2 of 14 animals reached a level of polymorphonuclear granulocytes of 1000 cells per cu mm in 4 days. Treatment was continued in 12 of these animals for 6 additional days and at the end of this time 10 of the 12 animals had 1000 or more granulocytes per cu mm of circulating blood,

the remaining 2 had levels of 800 and 950 cells per cu mm respectively.

Pathologic Findings The heart, lung, liver, kidney, spleen, adrenal and bone marrow of 26 rats fed thiourea were sectioned and examined microscopically. Of these organs only the thyroid glands, adrenal glands, bone marrow and spleen showed significant lesions. The thyroid hyperplasia and colloid depletion found have been previously described. Grossly the adrenal glands did not appear to be enlarged but were cherry red in color. Microscopically, the pathologic alterations present in these glands were hyperemia, often of extreme degree, hemorrhage, necrosis and lipid depletion. The hyperemia and hemorrhage, while commonly present in the inner cortex (reticularis), were not limited to this area. In a few adrenals, hyperemia or hemorrhage or both were present in the outer half of cortex and absent from the reticularis. Necrosis of adrenal cortical cells occurred in about one-half of the animals. The involved cells were scattered, less often grouped, in the outer one-third of the fascicularis. Rarely were they seen deeper in the cortex. Lipid depletion of moderate or marked degree was seen in all except 2 animals. This was studied in frozen sections stained with Sudan IV. In the majority, the depletion was usually greatest in, and often limited to, the outer half of fascicular and glomerular layers. Although measurements were not made, the cortex in a few adrenals was obviously reduced in thickness. The spleen of a number of rats showed marked hyperemia and lymphoid atrophy. The bone

* The crystalline product employed had been isolated from a fermentation residue and was furnished through the courtesy of Drs E L R Stokstad and B L Hutchings of Lederle Laboratories, Inc.

† Hutchings, B L, Stokstad E L R, Bohonos, N, and Slobodkin, N H. *Science*, 1944, 99, 371.

TABLE I
Recovery of Amino Acids from Protein Hydrolyzates

Protein Hydrolyzate	Amino Acid	Amino Acid Content* %	Amino Acid Added %	Total Content Found %	% Recovery
Casein	lysine*	7.3	7.0	14.3	100
"	"	7.8	7.0	14.6	97.4
"	"	7.34	7.0	13.9	94.0
"	"†	7.13	8.0	14.63	93
β Lactoglobulin	"	9.87	9.83	19.71	100
Casein	histidine*	3.25	3.0	6.15	97
"	"	3.11	3.0	6.17	101.9
Ghadin	"	1.99	2.0	3.89	95
Horse hemoglobin	"	7.63	7.6	15.3	99.1
"	"	7.72	7.6	15.4	101
Casein	arginine†	3.74	4.0	7.94	103
β Lactoglobulin	"	2.78	2.81	5.57	99.3
Casein	valine†	6.95	6.0	12.95	100
β Lactoglobulin	"	5.5	5.8	11.0	94.6
Gelatin	"	2.4	2.4	4.85	102

Percentages are uncorrected for moisture

* Determined with *L. mesenteroides*† Determined with *S. faecalis*TABLE II
Lysine Content* of Several Proteins

Sample	Lysine determined by										Other methods
	<i>Streptococcus faecalis</i>					<i>Leuconostoc mesenteroides</i>					
	%					%					
					Avg					Avg	
Casein (Labco)	7.83	7.39	7.34	7.76	7.6	7.50	7.54	8.0	7.48		8.34
						7.70	6.70			7.5	7.76
Gelatin (Knox)	5.2	5.2			5.2	3.98	3.75			3.87	5.86
Ovalbumin†	6.04	6.1	5.85		6.0	5.6				5.6	6.66
β Lactoglobulin†	10.4	10.0	10.4	10.4							
		10.6			10.4	10.5				10.5	11.16
Horse hemoglobin†	8.66				8.66	8.4				8.4	
Silk fibroin†	0.90	0.90			0.90	0.58				0.58	0.604
											0.726

* Percentages are expressed on the moisture-free basis

† Kindly supplied by the late Dr. Max Bergmann

The medium of McMahan and Snell¹ was modified for *Streptococcus faecalis* by doubling the phosphate concentration and by altering the vitamin supplement in the manner described by Hac *et al.*² The medium is very similar to that used by Stokes *et al.*⁶ The assay was carried out on a 2.5 cc scale incubated 16 hours at 30° C and growth measured turbidimetrically after dilution of each culture with 5 cc of saturated aqueous chlorothymol. Stokes *et al.*⁶ use 10 cc cultures, and follow growth titrimetrically after an incubation period of 40 hours. The determination of arginine, valine and lysine was investigated using *S. faecalis* as test organism. Growth response increased with increasing concentration over the following

ranges for the various amino acids: 8-50 γ *l*(+)-arginine monohydrochloride, 10-100 γ *dl*-valine and 20-100 γ *l*(+)-lysine monohydrochloride per 2.5 cc of medium.

Results. Recoveries of amino acids added to protein hydrolyzates were good (Table I). There were no drifts in assay values as dosage levels of samples were increased and assay values of protein samples tested at different times were reproducible (Tables II and III).

In Table II values for the lysine content of various proteins as determined with both *S. faecalis* and *L. mesenteroides* are given. Values for the histidine, arginine and valine content of proteins are given in Table III. Values obtained by other investigators are

and necrosis of the adrenals. Animals which receive, concomitantly, thyroxin injections or thyroid powder become granulocytopenic and leucopenic, while the incidence of anemia

and of adrenal hemorrhage and necrosis is greatly reduced. The granulocytopenia and leucopenia of these rats may be corrected by treatment with *L casei* factor.

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Microbiological Determination of Amino Acids IV Lysine, Histidine, Arginine, and Valine

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Microbiological assays for amino acids have been reported in a series of papers from this laboratory.¹⁻³ A considerable portion of our more recent work has been anticipated by published results from other laboratories,⁴⁻⁶ and we are therefore presenting only in abbreviated form additional methods for lysine, histidine, arginine, and valine and results of their application to 6 purified proteins: casein, gelatin, ovalbumin, β -lactoglobulin, horse hemoglobin, and silk fibroin.

Assay Procedures Samples for assay were prepared by autoclaving (at 15 lb steam pressure) 100 mg of the protein with 2 cc of 10% hydrochloric acid in a sealed tube for 24 hours. All assays were carried out as described by McMahan and Snell.¹

Leuconostoc mesenteroides was employed for the lysine and histidine assays. The medium of McMahan and Snell¹ was modified

for *L. mesenteroides* by increasing the phosphate content (Salts A) 8-fold and the arginine content 3-fold, and by altering the vitamin supplement (Solution 2) as described by Hac *et al.*²

The lysine assay was incubated 63 to 65 hours at 32 to 34° C. The standard curve was constructed from response of the test organism to graded amounts of *l* (+)-lysine in the range 5-50 γ per 25 cc of medium. Growth response was measured turbidimetrically after dilution of the contents of each tube with 5 cc of a saturated aqueous solution of chlorothymol. Growth response may also be measured acidimetrically[†] using the micro quinhydrone-calomel-electrode technic or with the glass-electrode assembly described by McQuarrie and Jones.⁷ When the assay tubes were to be titrated, 2% glucose was used instead of 1%, to permit formation of larger quantities of acid.

For the histidine assay, graded amounts of *l* (+)-histidine in the range 1-12 γ made up the standard curve. The culture tubes were incubated at 32 to 34° C for 72 hours. Shorter periods of incubation resulted in poor recoveries and drifting assay values.

[†] The folie acid requirement is based on a concentration with a potency of 40,000.

[‡] The values obtained for the lysine content (uncorrected for moisture) of a casein hydrolyzate assayed turbidimetrically and acidimetrically were 7.0 and 7.1%, respectively.

⁷ McQuarrie, E. B., and Konen, H. J., *Ind. Eng. Chem., Anal. Ed.*, 1944, **16**, 205.

* Present address: Department of Biochemistry, University of Wisconsin, Madison.

¹ McMahan, J. R., and Snell, E. E., *J. Biol. Chem.* 1944, **152**, 83.

² Hac, L. R., Snell, E. E., and Williams, R. J., *J. Biol. Chem.*, 1945, **159**, 273.

³ Hac, L. R., and Snell, E. E., *J. Biol. Chem.*, 1945, **159**, 291.

⁴ Dunn, M. S., Carmen, M. N., Shankman, S., Frankl, W., and Rockland, L. B., *J. Biol. Chem.*, 1944, **156**, 715.

⁵ Dunn, M. S., Carmen, M. N., Shankman, S., and Rockland, L. B., *J. Biol. Chem.*, 1945, **159**, 653.

⁶ Stokes, J. L., Gunness, M., Dwyer, I. M., and Caswell, M. C., *J. Biol. Chem.*, 1945, **160**, 35.

also given for comparison

Discussion Values for the lysine content of proteins obtained with *S faecalis* and *L mesenteroides* are in good agreement with each other and with values cited from the literature, except in the cases of gelatin and silk fibroin. The value obtained with *L mesenteroides* for gelatin appears to be low, and that for silk fibroin with *S faecalis* appears to be high when compared with the data cited. The value obtained for the lysine content of horse hemoglobin is in excellent agreement with that obtained by the isotope dilution method⁸.

The single value (2.8%) for the arginine content of horse hemoglobin probably should be disregarded since it is much lower than the values 3.4, reported by McMahan and Snell,¹ and 3.7, reported by Foster.⁸ The

⁸ Foster, G. L., *J. Biol. Chem.*, 1947, 159, 431

value (9.4%) for the valine content of horse nemoglobin is somewhat high as compared with the value, 8.8%, reported by McMahan and Snell.¹ Recent values for the histidine content of horse hemoglobin are unavailable. The agreement among other values reported in Table III is excellent.

Summary A method of assay for histidine and lysine employing *Leuconostoc mesenteroides* as test organism has been developed. This method is compared with those of Dunn *et al.*^{4,5} A method of assay similar to that of Stokes *et al.*⁶ for lysine, arginine, and valine using *Streptococcus faecalis* as test organism has been independently developed and studied.

With few exceptions, the data reported agree closely with those obtained by previous investigators with microbiological or isotope dilution methods.

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The Nature of Circulating Estrogen *

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In the course of investigations on the physico-chemical nature of circulating estrogen, we have found a large and constant portion of the latter to be closely associated with the blood proteins. Coincidentally, we have observed that blood estrogens will dialyze quantitatively past a collodion membrane.

Methods The methods used for the extraction of blood estrogens will be published in detail elsewhere. In brief, the procedure generally consisted of precipitation of the blood proteins with acetone. The supernatant fraction was combined with the acetone-ether washings of the precipitate, after which the two fractions were treated separately. In all instances, precipitation and washing of the protein was done in the cold.

After evaporation of acetone and ether

from the supernatant fraction, the latter was hydrolyzed with 4% H₂SO₄. The freed estrogen was then extracted with ether, and the water-washed extract was made up in a small amount of olive oil for subsequent assay.

The previously undenatured protein precipitate was subjected to partial alkaline hydrolysis with 0.1 N NaOH. After neutralization with CO₂, this material was extracted with ether and made up in olive oil.

Other methods of estrogen extraction included alcohol-ether precipitation, and ammonium sulfate precipitation followed by prolonged hot alcohol-ether Soxhlet extraction of the protein.

Portions of the same or similar bloods, as well as pure solutions of various natural estrogens, were also subjected to dialysis against distilled water through sausage casing (Visking, 25/32" diam). The dialysate

* Aided by grants from the Foundation for Applied Research and G. D. Searle & Co.

TABLE III
Histidine, Arginine, and Valine Content* of Several Proteins as Determined Microbiologically

Sample	Histidine†		Arginine		Valine		Values cited from the literature
	%	Avg	%	Avg	%	Avg	
Cysoen (Lobos)	3.3, 3.2, 3.2, 3.2, 3.2	3.2	3.82, 3.87, 3.53, 3.58, 3.60, 3.84	3.71	7.0, 6.5, 7.09, 7.09, 6.88	6.91	Histidine 3.15, 2.86 Arginine 3.96 Valine 6.76
Gelatin (Knox)	0.83, 0.83	0.83	8.73, 8.8	8.77	2.6, 2.6	2.6	Histidine 0.536 Arginine 9.16 Valine 2.76
Ovalbumin	2.28, 2.4, 2.35, 2.45	2.4	5.72, 5.79, 5.83, 5.50	5.7	7.07, 7.66	7.36	Histidine 2.30 Arginine 5.96 Valine 7.06
β Lactoglobulin	1.72, 1.8, 1.67	1.73	2.98, 2.95, 2.70	2.88	6.07, 5.75	5.91	Histidine 1.36 Arginine 2.86 Valine 5.56
Horse hemoglobin	8.2, 8.16, 8.13, 8.2, 8.06	8.2	2.8	2.8	9.0, 9.8	9.4	
Silk fibron	0.41, 0.4	0.41	0.99	0.99	3.36, 3.46	3.41	Histidine 0.345, 0.416 Arginine 1.116 Valine 3.56

Histidine was determined with *L. mesenteroides*, arginine and valine were determined with *S. faecalis*.
* Values are expressed on the moisture free basis.

† Employing the organism *L. delbrueckii* 3 and a slightly different medium the values obtained for the histidine content of cysoen and gelatin—3.2 and 0.84, respectively—are in excellent agreement with those above.

TABLE II
Dialysis of Pure Estrogens

Estrogen	μg inside before dialysis	Form	Hrs of dialysis	μg found in	μg found out	% recovery	% dialysis*
α Estradiol	10	Nr Salt	24	4.0	6.4	104	74
	10		48	1.0	8.0	90	94
	1.0	"	48	—	0.9	—	105
"	9.5	Free	48	4.5	4.0	90	35
"	2.0	"	48	—	0.4	—	25
β Estradiol	10	Nr Salt	48	—	8.3	—	100
Estrone	2.0	"	48	—	1.8	—	106
Estril	30		48	—	24.8	—	98

* Based on theoretical considerations with respect to volume ratios across the collodion membrane

was ether-extracted before and after acid hydrolysis and prepared for injection in olive oil. The material inside the cellophane bag was often analyzed for estrogen coincidentally in the case of blood, the acetone procedure outlined above was employed. In all instances, dialysis was carried out in the cold (2°C) in a mechanical shaking device.

Biological assay for estrogenic activity was by a modification¹ of the 6-hour uterine-weight method of Astwood.² The index employed was the increase in the ratio of uterine weight to body weight in immature female rats. Most of the results were represented arbitrarily in terms of alpha-estradiol equivalents.

Results Blood and sera obtained from normal and pregnant cows, normal and gonadotrophin-injected rabbits, and pregnant women were analyzed for estrogen.

It was uniformly found that only one-third of the total estrogen was present in the acetone supernatant fraction from which the protein had been removed without denaturation (Table I). This portion was apparently almost entirely present in a conjugated form (probably as an ester), since it could be extracted with ether only after acid hydrolysis. The remainder, or two-thirds, existed associated with the protein in a form liberated by weak alkaline hydrolysis. Subsequent hydrolysis at pH 1 did not increase the yield. It may be noted that only very small amounts of estrogen were present in all instances.

In the course of related studies it was

found that pure estrogens will dialyze quantitatively past a collodion membrane. This is illustrated in Table II. Among the estrogens studied, free estradiol (which we have not found to any significant degree in blood) dialyzes too slowly to reach equilibrium in a reasonable length of time. This may be due to its hydrophobic nature in a salt-free environment. Otherwise, dialysis appeared to reach theoretical completion in about 48 hours. Dialysis was not complete in 24 to 26 hours. This is illustrated by the incomplete dialysis of the sodium salt of alpha-estradiol after 24 hours (Table II) and of pregnant cow plasma and laked cells after 26 hours (Table III). Similar samples had dialyzed completely after 36 to 48 hours.

Table III shows that the amount of estrogen obtained from a 36 to 72 hour dialysate of blood was approximately equivalent to that found by exhaustive chemical extraction procedures (Table I). The optimal volume ratio of material inside the collodion bag to that outside was found to be about 1.5. Dialysis was incomplete where this condition was not met, as in the case of the whole blood sample from the gonadotrophin-injected cow (Table III). In this case, the volume ratio was only 1.2.

Discussion Previous studies on natural blood steroids having hormonal activity have almost uniformly neglected the protein fraction in spite of the fact that a large amount of cholesterol is found closely associated with the latter.³ However, a variable amount of estrogen has been found associated with

¹ Pincus, G. and Werthessen, N., personal communication.

² Astwood, E. B., *Endocrinology* 1938, 23, 25.

³ Tronsgaard, N., and Koudahl, B., *J. Physiol. Chem.* 1926, 153, 111.

TABLE I
 Distribution of Blood Estrogen

Sample	Method	Volume cc	Fraction	Treatment of protein before ether extraction	Estrogen as μg estradiol per 100 cc	% estrogen in protein fraction
Rabbit* Serum	Acetone Pptn	72	Supernatant Protein	—	<0.34	Total 0.91
				Alkaline partial hydrolysis	0.47	>58
Cow** Whole blood†	" "	100	Supernatant Protein	—	0.10	Total 0.30
				" "	0.20	
Pregnant cow (6 mo) Whole blood†	" "	100	Supernatant Protein	—	0.15	
				(1) Aqueous extract	0	
				(2) Alkaline partial hydrolysis	0.23	61
				(3) Hydrolysis at pH 1	0	
Pregnant cow (6 mo) Whole blood†	Alcohol ether Pptn	65	Supernatant Protein	—	0.12	Total 0.36
				Alkaline partial hydrolysis	0.24	67
Pregnant human (7 mo) Whole blood†	(NH ₄) ₂ SO ₄ Pptn	100	Supernatant Protein	—	0.20	Total 0.60
				Soyablet†	0.40	67

* Gonadotrophin treated

† Preserved with potassium oxalate 0.2%

‡ Continuous extraction with hot alcohol ethyl for 48 hours

15260 P

Effect of Certain Amino Acids on Healing of Experimental Wounds of the Cornea

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As a non-vascularized tissue, the cornea is unique from a biological point of view. It receives its nourishment by diffusion of fluids through the entire corneal substance. In trauma this diffusion may be disturbed leading to vascularization and loss of transparency.

Earlier many attempts were made to nourish the elements of tissue cultures and to increase their rate of proliferation *in vitro*¹. The attempt to influence the regeneration of corneal defects by embryonic tissue extracts encountered many difficulties. Fischer² found that in tissue cultures *in vitro* the embryonic extracts could be replaced by an artificial medium in which amino acids furnished the building stones for the synthesis of protoplasm. Cystine, which seemed to function both as an energy-furnishing ingredient and as a growth catalyst, apparently played a major role in this medium. This special function of cystine is probably connected with its sulfur content, as free sulfhydryl groups often activate proteolytic systems in the process of growth.

Encouraged by Fischer's results the writer has made an attempt to influence the regeneration of experimental wounds of the cornea by administration of amino acids to this tissue *in vivo*. For the first experiment 36 guinea pigs were used. The technic employed was a modification of the method of Gundesen and Liebman³. Superficial vertical incisions were made across the cornea into the epithelium of both eyes by means of a Castroviejo double knife with blades 2 mm apart. The cornea was immediately stained with fluorescein and the epithelium between

the two lines evenly abraded with a spatula. The corneae were periodically stained and the fluorescein-positive areas registered on a chart drawn on logarithm paper. The chart consisted of a ring symbolizing the limbus with a vertical double line in the center. By counting the number of stained and unstained squares of the paper, a quantitative expression of the healing process could be obtained.

In 18 of these animals (1,a) the amino acid solution was dropped into the right eye every hour while the left control eye received a corresponding amount of physiological saline. In the remaining 18 animals (1,b) an ointment containing the amino acids was applied twice a day to the right eye together with a 5% boric acid ophthalmic ointment which was applied to both eyes.

The same experiment was repeated on 6 other animals (2) but instead of a stripe, a round erosion, 3.5 mm in diameter, was made with a trephine in a similar manner. The amino acid solution was made in 6% NaCl and contained 2 mg cystine, 5 mg proline, 6 mg asparagine and 14 mg glutamine per ml, and was adjusted to pH 7.2. The ointment was prepared by emulsifying 70 g aquafor with 30 ml amino acid solution which contained the aforementioned amino acids in 10-fold concentration*.

A complete regeneration of the corneal defect in the eyes treated with amino acids was achieved within 12 to 42 hours (in less than 30 hours in an average), while the healing process in the control eyes required 55 to 120 hours.

In a third series of experiments the effect of amino acids on healing of deep non-per-

¹ Biseeghe, V., and Schaeffer, A. J., *Die Gewebe zuchtungen in vitro*, Springer, Berlin, 1928.

² Fischer, A., *Acta Physiol. Scandinav.*, 1940, **2**, 143.

³ Gundesen, T., and Liebman, S. D., *Arch. Ophthalm.*, 1944, **31**, 29.

* I am indebted to Dr. Ernest Geiger for suggestions and for the material used in these experiments.

TABLE III
Dialysis of Estrogen in Blood

Sample	Volume cc	Duration of dialysis in hrs	Estrogen as μg α Estradiol per 100 cc
Cow serum	160	48	0.25
Cow*			
Whole blood†	200	46	0.20
Plasma†	100	46	0.40
Laked cells†	100	46	0.35
Pregnant cow (6 mo)			
Whole blood†	50	36	0.34
Plasma†	100	26	0.10
Laked cells†	100	26	0.20
Pregnant human			
Whole blood† (3 mo)	100	72	0.22
Whole blood† (5½ mo)	100	72	0.55

* Gonadotropin treated

† Preserved with potassium oxalate 0.2%

the protein in mare serum⁴ and in human pregnancy serum.⁵ This bound estrogen could be liberated by prolonged strong acid hydrolysis, but not by refluxing 4 hours with alcohol, nor by ultrafiltration or tryptic digestion.

Our data suggest that an equilibrium exists in blood between estrogen and protein. Thus, two-thirds of the total estrogen is normally closely associated with the protein. The remainder is apparently present in an esterified form in the aqueous phase. The hydrophilic nature of this esterified estrogen may be involved in the formation of the protein complex. It is improbable that the latter represents merely simple adsorption, since the relative amount of estrogen found associated with the protein by different extraction procedures is quite constant (Table I).

The estrogen-protein complex is of such a nature that dialysis results in its progressive dissociation. It is probable that this is accomplished by preserving the ratio between dissociated and protein-bound estrogen inside the collodion bag. As dialysis proceeds, gradually removing the protein-free estrogen, further dissociation of the estrogen-

protein complex is permitted. This postulated equilibrium, then, seems to obey the law of mass action, enabling dialysis to proceed toward completion, depending upon the concentration gradient of estrogen across the membrane.

The above results bear on the fundamental problem of steroid transport and activity *in vivo*, and suggest that estrogen bound to protein is potentially available for physiological action by dissociation at the cell membrane.

Summary The total estrogen content of blood and sera obtained from normal, pregnant and gonadotrophin-injected animals and women was uniformly low. Values approximating 0.5 μg of alpha-estradiol equivalents per 100 cc of blood were found.

Two-thirds of the total estrogen was closely associated with the protein fraction. The remaining one-third existed mainly in a hydrophilic, non-protein conjugated form.

Blood estrogens, as well as pure solutions of the sodium salts of estrone, alpha- and beta-estradiol, and estriol, were shown to dialyze quantitatively past a collodion membrane.

The authors wish to express their indebtedness to Dr. Samuel C. Gwynne and to Mr. Raymond Umbrugh for furnishing them with some of the blood samples.

⁴ Hrussov, E. P., *Festschrift E. C. Barrell*, 1936, 327 (Basel).

⁵ Rakoff, A. E., Paschalis, K. E., and Cantarow, A., *Am. J. Obstet. and Gynec.* 1943, **46**, 856.

The danger of air embolism during operations on the neck and chest, because of negative intra-thoracic pressure, is well known¹ though possibly not fully appreciated and we shall show that this danger also exists for operations on the skull. The experimental and clinical literature contains numerous reports of air embolism following the injection of air or oxygen into various cavities and spaces of the body². It will be further shown that fibrinous masses may develop acutely in the heart during air embolism, this observation has only been reported once before as far as we know. Richardson, Coles and Hall³ observed occasional flecks of fibrinous material around the chordae tendineae of the right ventricle, and in 3 cases saw a friable clot of blood and air that possibly acted as a ball valve against the pulmonary artery, these investigators used nembutalized dogs and injected air into a femoral vein.

Although Kleinschmidt⁴ has published an excellent experimental study of air embolism in rabbits, we shall report our own findings because Kleinschmidt and other experimenters injected air intravenously, while our work deals with air embolism following operations on the skull and spinal region.

Technic. The rabbits were generally narcotized by the subcutaneous injection of 150 mg of sodium barbital per kilo and operated 30 to 45 minutes later, in a few instances morphine or ether was used. The chief operations were tracheotomy, exposure of the thoracic spinal column and cord, transection of the cord combined at times with pithing below the site of transection, exposure of the diploic veins in the parietal bones or removal of the outer lamella of the squamous portion of the occipital bone. In some series,

the vagi were sectioned in the neck, in others they were left intact. Inspiratory dyspnoea was often produced for short periods by an ordinary flap-valve slipped over the tracheal cannula. In the curare experiments, artificial respiration was given at the rate of 30 per minute. The blood pressure was recorded by a mercury manometer in a number of experiments.

If the animal succumbed acutely, an autopsy was carried out at once, otherwise a biopsy was performed 30 to 55 minutes after the operative interference.

Experimental Results. *Cord operations* were carried out on 28 rabbits. In 22 both vagi were cut and 11 died acutely, 8 of these 11 rabbits revealed air bubbles in the pulmonary artery, the remaining 11 rabbits were biopsied and one showed both air bubbles in the pulmonary artery and a good amount of fibrin in the right ventricle, a second rabbit revealed no air bubbles but both ventricles showed some fibrin shreds. In 6 rabbits, the vagi were left intact, none of these died acutely but a biopsy revealed air in the pulmonary artery of 2. Thus 11 out of 28 rabbits showed air bubbles in the pulmonary artery.

Skull operations were performed on 9 rabbits and the vagi were cut in all. Acute death occurred in 4 and 3 of these showed air bubbles in the pulmonary artery. Biopsy was necessary in 5 and 2 of these subacute cases showed air bubbles in the pulmonary artery. In one interesting experiment, both vagi cut, air bubbles were seen coursing through the exposed diploic veins of a parietal bone during deep inspiration. Biopsy was performed 27 minutes after the air was seen entering the diploic veins. At that time, the blood pressure was 80 mm Hg and the respiration 172 per minute. On opening the chest in the midline, the heart was beating powerfully and regularly, the pulmonary artery was distended by air bubbles and the right side of the heart was fuller than the left. Section of the heart revealed no air bubbles in the right atrium and ventricle, but the right atrium contained a small amount of fibrin while all other chambers were free of fibrin. It seems probable that this rabbit

¹ Ceelen, W., in *Henke Lubarsch Handbuch der speziellen pathologischen Anatomie und Histologie*, Berlin, Julius Springer, 1931, III, Part 3, 119.

² Reviews of experimental and particularly clinical cases. Brevfogle, H. S., *J. A. M. A.*, 1945, 129, 342; Wolffe, J. B., and Robertson, H. F., *Ann. Int. Med.*, 1935, 9, 162.

³ Richardson, H. F., Coles, B. S., and Hall, G. E., *Canadian Med. Assn.*, 1937, 36, 584.

⁴ Kleinschmidt, O., *Arch. f. Klin. Chirurgie*, 1915, 106, 782.

TABLE I

Summary Table Showing Type of Corneal Injury and the Average Healing Time in Control and Amino Acid treated Animals

Expt No	Experimental injury	Treatment		No of Animals	Healing time*	
		Control	Amino acid treated		Control eye	Treated eye
1,a	Gunnar pigs Vertical 2 mm erosion	Physiol saline hourly	Amino acid soln hourly	18	64(55 108)	22(15 32)
1,b		5% boric acid ointment twice daily	5% boric acid ointment containing amino acids twice daily	18	78(66 120)	26(12 42)
2	Round erosion, 3.5 mm	physiol saline hourly	Amino acid soln hourly	6	62(50 112)	21(14 38)
3,a	Rabbits Deep non perforating trephine wound	" "	" " "	2	95(86 104)	35(28 42)
3,b		5% boric acid ointment twice daily	5% boric acid ointment containing amino acids twice daily	10	108(96 120)	40(32 56)

* The first figure gives the average and the values in parentheses the minimum and maximum values of the individual experiments

forating trephine wounds of the cornea was examined. In 12 rabbits a central defect 2.5 mm in diameter and 0.4 mm deep was made into the substance of the cornea of both eyes. The right eye was treated in 2 animals (3,a) with hourly administration of drops containing amino acids while the left eye received saline solution. The other animals received ointment applications as in the first experiment (3,b).

In the eyes treated with amino acids, the corneal defect was covered with epithelium within 32 to 56 hours and in the control eyes between the 4th and 5th day. The biomicro-

scopic parallel with the histological examination showed that in the amino acid-treated eyes the same phase of the healing process is reached after 2 days which the control eyes reached only after the 6th day of experiment.

The results of treatment of over 100 clinical cases in man with the same ointment will be published elsewhere. Although it is difficult to evaluate these data quantitatively since the ointment was never applied alone, it would appear that the application of amino acids accelerates the healing of corneal wounds particularly when ulcerative processes are involved.

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Accidental Air Embolism and Fibrin Formation in the Heart of Rabbit

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During operations on the spinal column, cord and skull, a number of rabbits died more or less acutely and their death was at first attributed to that diffuse symptom-complex "vascular shock." Autopsies, however, soon

revealed cleaner factors that played an undoubted rôle in the fatal outcome, gas bubbles were seen in the pulmonary artery associated at times with fibrin formation in the heart, especially in the right ventricle.

fibrin networks particularly in the right ventricle. In our series, 7 rabbits showed fibrin networks in the heart without any detectable air bubbles.

It seems obvious that this fibrin must have been formed by churning of the air-blood mixture in the right ventricle during cardiac activity. Why every heart with air in the pulmonary artery did not show fibrin is probably due to a number of factors. If the air bubbles are promptly expelled into the pulmonary artery, no defibrination of the right ventricular blood can occur, and air in the pulmonary artery does not measurably obstruct the flow of blood according to Kleinschmidt (⁴ p. 802). The longer the right ventricle is able to churn the blood-air mixture the greater the chance of causing defibrination. If the amount of air is too large, right ventricular failure ensues, as is demonstrated by the bulging dark-colored right atrium and ventricle and the contracted, pink, and empty left atrium and ventricle. Posture of the body also is important, Kleinschmidt (⁴ p. 791) states that air is expelled with greater difficulty when the apex of the heart is at a higher level than the base. Another factor is the coagulability of the blood. We attempted to correlate the coagulation times before and after the experiment with the appearance of fibrin in the heart, but our results in 10 experiments permitted no definite conclusion.

Our data do not permit a definite statement about the rôle played by section of the vagi in the production of air embolism because the number of controls with vagi intact is inadequate, it seems, however, that section of the vagi is not a vital factor. Acute death, however, was more frequent in the

cord series of 28 rabbits when the vagi had been cut.

Summary 1 In barbitalized rabbits subjected to operations on the spinal column, cord and skull, air embolism is not a negligible danger. 14 out of 37 rabbits showed air bubbles in the pulmonary artery. Acute death after such operations in the rabbit must not be diagnosed as "vascular shock," tacitly implying a nervous component, until a careful autopsy has excluded air embolism.

2 Air bubbles were observed entering and coursing through exposed sections of the diploic veins of the parietal bone during inspiration.

3 Absence of air bubbles in the right heart and pulmonary artery does not exclude air embolism for the air may have been absorbed. The diagnosis of air embolism may still be made if fibrin is found in the heart. Fibrin formation in the heart, especially the right ventricle, was seen 6, possibly 7, times when no air bubbles were detectable anywhere. Occasionally a slight amount of fibrin may also be detected in the left ventricle. Here apparently some air bubbles passed through the pulmonary circuit and entered the left ventricle. Fibrin may occur only in the left ventricle; this was seen once when artificial respiration ruptured lung aveoli and air entered the left heart and arterial system only.

4 No definite evidence of pulmonary infarction was seen in the lungs on macroscopic and microscopic examination. It seems probable that pulmonary infarction would have occurred had some of the animals been allowed to survive for longer periods of time.

5 Pulmonary edema was slight in the majority of our experiments.

would have recovered

In this series, 5 out of 9 rabbits showed air bubbles in the pulmonary artery

Cord operations under curare and artificial respiration were studied in 16 barbitalized rabbits, the vagi were cut in 6. In this series, only one rabbit out of 16 showed air bubbles in the pulmonary artery and this exception was probably caused by imperfect curarization. No fibrin was found in any heart

Fibrin Networks in the Heart In a number of rabbits dying acutely or biopsied 30 minutes more or less after the operative procedure, a fibrin network was noted in the right ventricle, though it also occurred in the right atrium and sometimes in the left ventricle. The amount of fibrin varied considerably. In one extreme instance the elastic, reddish or reddish-black network interlaced the papillary muscles and chordae tendineae of the right ventricle practically filling this cavity and a portion extended up to the pulmonary artery, in most instances, however, the amount was much less. When fibrin occurred in the left ventricle, it was present only as a thin film

In the series of 16 acutely fatal cases, 10 revealed fibrin in the heart, in 2 or 3 of these 10 fibrin-containing hearts, no air bubbles were detected, but air bubbles were seen in the other 7 hearts. The vagi had been cut in all 16

In 21 subacute experiments, fibrin was found in the heart of 7 cases and in 4 of these no air was seen in the pulmonary artery, the remaining 3 showed air in the pulmonary artery. In 3 instances, fibrin was found in both ventricles without any sign of air. One rabbit (vagi intact) showed air bubbles but no fibrin, the remaining 13 rabbits (vagi cut in 9, intact in 4) showed neither air nor fibrin in the heart

An instructive accident demonstrated that air embolism could produce the formation of fibrin in the left ventricle only. This happened when the lung alveoli were ruptured during artificial respiration. Immediate autopsy showed air bubbles in the aorta, carotid and mesenteric arteries, but no air was seen in the right side of the heart and pulmonary

artery. In this heart, the left ventricle showed fibrin while the right ventricle contained none

Since the right ventricle occasionally contained definite fibrin networks, it might be assumed that particles could be dislodged and finally plug some of the small subdivisions of the pulmonary artery. No definite signs of embolism, however, were detected in the lungs of rabbits that died acutely or that were biopsied although these animals showed fibrin in the right ventricle. Very frequently section of the various lobes of the lung showed reddish to red-black polygonal areas that were a few millimeters in diameter, these congested areas were surrounded by larger, grayish-white areas that were full of air and were largely bloodless. Microscopic sections also gave no evidence of embolism. The time, however, between the formation of fibrin and acute death or biopsy was probably too short to permit adequate stasis for infarction to occur

Pulmonary edema in the great majority of our experiments was slight and limited at times to one lobe. This is contrary to Kleinschmidt's results (⁴ p 798) who always found strong pulmonary edema, Richardson, Cole and Hall also report marked pulmonary edema (³ p 587)

Discussion The recorded experimental results show unmistakably that air embolism during operations on the spinal column, cord and skull in heavily barbitalized rabbits is an ever-present danger and that this air embolism may cause acute death or a subacute state from which recovery probably could ensue. Thus in 37 spinal and skull operations, 16 rabbits revealed air bubbles in the pulmonary artery. It is therefore clear that the diagnosis "shock" as the cause of death in rabbits after operations on the skull, neck and chest should not be made until a careful autopsy has determined whether or not air embolism was the primary factor. This diagnosis, however, is rendered more difficult by the fact that the air bubbles may have been absorbed before the autopsy was made. Here a practically unknown sign is at times available that indicates the previous existence of air emboli and that is the presence of

the generalized spasticity so common in acute poliomyelitis. It is finally obvious that the presence of lesions in the motor cortex and in brain stem centers which function to modify muscle activity, has occasionally suggested that these lesions are sources of interference with inhibitory motor mechanisms.

The difficulties of analysis of the pathogenesis of spasticity in humans can be partly overcome in rhesus monkeys because of a fortunate circumstance. We have shown that in rhesus monkeys inoculated intranasally or intracerebrally, the progress down the brain stem of lesions produced by virus activity may occasionally be arrested spontaneously at any point before reaching the spinal cord.^{10, 11} Such non-paralytic attacks of poliomyelitis in rhesus monkeys are not accompanied, in our experience, by severe spastic phenomena, but as a rule the lesions in the brains of such animals are not severe. However, brain lesions may be very severe in some inoculated animals in the preparalytic period, when spasticity is the predominant neurological symptom. It was thought possible that in some animals in this stage, virus activity or lesions might not have progressed as far as the spinal cord. With severe infections induced by most of the virus samples we have used, this is rarely true, since lesions are usually found to be present in both the lumbar and the cervical cord by the time that spasticity is well-marked.[†] With the Lansing strain,¹² however, we have found that not only do the arms usually show paralysis first but, in addition, the onset of paralysis in the legs is often somewhat delayed as compared with

other strains.[‡] Consequently, an attempt was made to kill certain animals inoculated intracerebrally with Lansing virus in the preparalytic period after definite spasticity had become manifest. It was found in 2 cases that marked spasticity and hyperreflexia were present in arms and legs *before* lesions or virus (in one case) could be demonstrated in the lumbo-sacral cord, although lesions and virus activity were present in the brain and cervical cord. An additional animal which had been inoculated with the Rockefeller MV strain in 1939 and had been killed in the preparalytic period for another purpose was subsequently found to show a similar pathological picture. It was thus apparent that lesions in the brain alone could produce the spasticity of acute poliomyelitis.

The protocols of these animals are of sufficient importance to be presented here.

Rhesus B474 This was an adolescent rhesus monkey in good condition.

Nov 7, 1945 Under nembutal anaesthesia a trephine hole was prepared over the central sulcus in the midline, and 0.4 cc of a 10% rhesus spinal cord suspension containing the Lansing strain of virus was inoculated into each dorsal thalamus.

Nov 12 No abnormal signs were noted.

Nov 13 The animal was observed to be very awkward and stiff in climbing and running. Extensors and flexors of arms and legs were tense and abnormally resistant to passive movement. Deep reflexes of arms and legs were hyperactive. Head tremor was present. No weakness or difference in strength between opposite limbs could be detected by careful examination.

¹⁰ Bodian, D., and Howe, H. A., *Bull. Johns Hopkins Hosp.*, 1941, **69**, 135.

¹¹ Bodian, D., and Howe, H. A., *Bull. Johns Hopkins Hosp.*, 1945, **76**, 1.

[†] Six rhesus monkeys killed while showing generalized spasticity in the preparalytic period after inoculation with one of 3 virus strains recently isolated from humans (Sudeck, 1941; Riley, 1943; Frederick, 1945) were found to have poliomyelitic lesions in both cervical and lumbar cord segments.

¹² Armstrong, C., *Pub. Health Rep.*, 1939, **54**, 2302.

[‡] This curious strain characteristic can be illustrated by the fact that 61 of the last 81 rhesus monkeys inoculated intrathalamically with Lansing virus in this laboratory were first paralyzed in one or both arms, whereas only 6 were first paralyzed in one or both legs, and the remaining 14 exhibited both arm and leg weakness on the first day of paralysis. In contrast, only 17 of 82 monkeys inoculated similarly with one of 6 other strains isolated in this laboratory were first paralyzed in one or both arms, 20 were first paralyzed in one or both legs, and 45 had both arm and leg weakness on the first day of paralysis.

Experimental Evidence on the Cerebral Origin of Muscle Spasticity in Acute Polomyelitis *

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One of the common symptoms of acute polomyelitis in man and in experimental primates is the muscular rigidity, associated with hyper-reflexia, which often precedes flaccid paralysis. The considerable recent interest in this phenomenon has been associated with conflicting views as to its pathogenesis, its possible role in producing chronic deformities, and its appropriate treatment. Recent observations specifically directed at determining the characteristics and distribution of muscle spasticity in acute human polomyelitis are in agreement with respect to the following points: 1 Muscle spasticity in acute polomyelitis is a reflex phenomenon associated with increased stretch reflexes.¹⁻⁸ 2 Spasticity in acute polomyelitis has a widespread occurrence in the skeletal musculature, may be present in both flexors and extensors, and may occur in partly weakened muscles as well as in muscles of normal strength.^{1,2,4,6} Our observations in the rhesus monkey are in agreement with these findings in human subjects, although

in some monkeys spasticity is not observed. This may result because of absence of the causes of this symptom, because of failure of detection, or because severe motoneuron paralysis occurs so quickly as to shorten greatly the period of spasticity or to preclude its occurrence. In some monkeys pre-paralytic spasticity may be so severe that awkwardness of gait and posture is apparent, and the resistance of extremities to passive movement is claspknife in character. At this stage a fine tremor may be present.

The fact that the lesions of paralytic polomyelitis occur in a number of nervous centers concerned with muscle function in the brain as well as in the spinal cord, makes possible a variety of hypotheses regarding the pathological site of origin of this symptom. Added to the difficulty of analysis imposed by the widespread nature of lesions usually present in fatal human cases, is the fact that the symptom in question has usually disappeared by the time death occurs. In spite of these difficulties, it is most commonly assumed that the lesions which are responsible for the symptom of spasticity are those in the region of the spinal cord which supplies the local reflex mechanism.^{3,6,8,9} Others attribute the hyperirritability of the stretch reflex to possible irritation of the muscle, nerve, posterior roots, or meninges by the virus,⁴ or to lesions of sensory neurons in the posterior horns as well as in sensory ganglia.⁷ None of our observations on pathological changes in many human or monkey specimens has suggested to us that lesions in these locations are quantitatively sufficient to be of primary importance in producing

* Aided by a grant from The National Foundation for Infantile Paralysis, Inc.

¹ Schwartz, R P, and Bouman, H D, *J A M A*, 1942, **119**, 923.

² Bouman, H D, and Schwartz, R P, *N Y State J Med*, 1944, **44**, 147.

³ Schwartz, R P, Bouman, H D, and Smith, W K, *J A M A*, 1944, **126**, 695.

⁴ Withins, A L, Brazier, M A B, and Schwab, R S, *J A M A*, 1943, **123**, 188.

⁵ Brazier, M A B, Withins, A L, and Schwab, R S, *New England J Med*, 1944, **230**, 185.

⁶ Moldaver, J, *J A M A*, 1943, **123**, 74.

⁷ Moldaver, J, *J Bone and Joint Surg*, 1944, **26**, 103.

⁸ Kabot, H, and Knapp, M E, *J A M A*, 1943, **122**, 989.

⁹ Kabot, H, and Knapp, M E, *J Pediat*, 1944, **24**, 123.

TABLE II
Subinoculation Tests of Suspensions of Anterior Quadrants of Spinal Cord Enlargements of Rhesus B430 (Preparalytic Poliomyelitis)

		Deaths or paralysis among inoculated animals			
Virus Dilution		Rt Cervical	Lt Cervical	Rt Lumbar	Lt Lumbar
Mouse Tests	10-1	11/16	7/8	0/8	0/8
	10-1 5 and 10-2	8/16	12/16	0/8	0/8
	10-2 5 and 10-3	4/14	4/14	0/8	0/8
	10-4	1/16	1/16	0/8	0/8
Monkey Tests					
	10-1			0/1	0/1

|| The mouse titrations were part of a larger study of virus levels in infected monkeys being carried out in collaboration with Dr. Mary Cumberland. In this study it was found that intracerebral inoculation in rhesus monkeys was a more sensitive test for the presence of Lansing virus in monkey cord than was mouse inoculation. The monkeys shown in this table were inoculated intrathalamically and killed after three weeks. Sections of cord and brain were examined and found normal. Not enough material was available for additional monkey tests.

and sparse at C8 and T1. Neuronal changes were found only on the right side in segment C7. These consisted mostly of various degrees of chromatolysis with only about 4% of all cells on the left side destroyed.

Complete serial sections of the lumbar cord segments L3 to L7, and S1, were stained and examined. No lesions of poliomyelitis were found.

Every 20th section of the brain was stained and examined. A typical poliomyelitic distribution of moderate to severe perivascular and focal infiltrative lesions and of neuronal destruction was found. A predominance of severe lesions was observed on the right side of the brain stem, especially in globus pallidus, subthalamic centers, midbrain tegmentum, reticular formation, and vestibular nuclei. Lesions were not observed in the left cerebral cortex or thalamus.

Summary of Rhesus 959 This animal was of interest in that the only preparalytic symptoms noted were fever, and spasticity and increased knee tendon reflex of the right leg. Lesions were absent in the lumbar cord, and in the left motor cortex, but were severe on the right side of the brain stem, especially in the globus pallidus, subthalamic centers, midbrain tegmentum, reticular formation, and vestibular nuclei. If cortical involvement had been responsible for spasticity of the right leg, lesions would have been expected on the left side, but none were found there. The right-sided unilaterality of spasticity in this case is suggestively asso-

ciated with ipsilateral predominance of lesions in the brain stem.

Rhesus B430 This was an adolescent animal in good condition.

Aug 30, 1945 Under nembutal anaesthesia a trephine hole was prepared over the central sulcus in the midline, and 0.4 cc of a 5% rhesus cord suspension containing the Lansing strain of virus was inoculated into each dorsal thalamus.

Sept 4 Rectal temperature had risen to 105°F from a baseline of about 104°.

Sept 5 The animal now was very awkward and stiff in running and climbing, and had marked head tremor. Palpation revealed that arms and legs were very strong, but very resistant to passive manipulation. All muscle groups appeared to be tense, but especially the forearm flexors and the hamstring muscles showed rigidity of the clasp-knife type. Tendon reflexes were exaggerated.

Under ether anaesthesia, the animal was exsanguinated from the femoral arteries. With sterile precautions, the cervical and lumbar enlargements of the spinal cord were removed separately for virus assay. The animal was then perfused through the aorta with 10% formalin containing 1% acetic acid. The brain and remainder of the spinal cord were removed for histological study.

Results of Virus Assays Right and left anterior quadrants of cervical and lumbar enlargements were prepared separately as 10% aqueous suspensions. Subinoculations were done in mice and in rhesus monkeys, with

TABLE I
Comparison of Numbers of Normal and Pathological Motoneurons in Cervical Enlargement of Rhesus B474, Based on Counts of Lateral Columns of 45 Equally Spaced 15 μ Sections (Preparative Polomyelitis)

	Left	Right	Expected on basis of studies of normal controls
Normal	400	314	450 500
Chromatolytic	44	93	—
Necrotic	9	16	—
Estimated deficit [§]	0 47	27 77	—
Total	453 500	450 500	450 500

[§] Motoneurons absent because of complete autolysis or phagocytosis

Under ether anaesthesia, the animal was perfused through the aorta with 10% formalin containing 1% acetic acid. The brain and spinal cord were removed for histological study. Serial sections at 15 μ were prepared of the cervical cord from C6 to T1 inclusive, and of the lumbosacral cord from L2 to S1 inclusive. All sections of the above 8 lumbosacral segments were stained with gallocyannin and examined.

Histopathological Findings Sections of the cervical cord contained typical early lesions of poliomyelitis, with moderate perivascular and diffuse leucocytic infiltration, and neuronal changes. Relatively few neurons were destroyed, but a considerable number showed various degrees of chromatolysis. The left anterior horn was almost free of lesions except in the lower part of C8 and the upper part of T1. Actual motoneuron destruction on the right side was limited to segment C7, although various degrees of chromatolysis were present in all cervical segments. An actual count of the anterior horn cells in 45 sections taken at intervals through the cervical enlargement showed the distribution of normal and affected motoneurons seen in Table I.

A quantitative study of the relationship of motoneuron destruction to muscle weakness, now in progress, indicates that the amount of motoneuron damage in the cervical cord was far below the level which produces clinically evident weakness.

All sections of the lumbosacral enlargement (L2 to L7, and S1) were examined. No lesions of poliomyelitis were detected.

The brain was imbedded in celloidin and sectioned at 60 μ . Every 20th section was stained with gallocyannin and examined. A

typical distribution of severe focal and diffuse infiltrative lesions and of neuronal destruction was found. Severest lesions were observed in the motor cortex (areas 4 and 6 of Brodmann), dorsal thalamus, fields of Forel, substantia nigra, superior colliculi, midbrain tegmentum, reticular formation of hindbrain, vestibular nuclei, and basal cerebellar nuclei. Neuron destruction, however, was most marked in the reticular formation and in the large cells of the vestibular nuclei.

Rhesus 959 This was an adolescent female, slender but active.

March 17, 1939 Under ether anaesthesia a trephine hole was prepared over the right motor cortex and 0.2 cc of a 20% rhesus cord suspension containing the Rockefeller Institute MV strain of virus was injected in the cortex of the leg region.

March 23 Rectal temperature had risen from a base-line of 102°F on March 21 to 105.2°F. No muscular weakness was detected by observation of movements or by palpation. The right leg, however, was observed to be definitely more spastic than the left leg or arms, and the right knee jerk was more active than the left. Under ether anaesthesia, the animal was perfused through the aorta with 10% formalin containing 1% acetic acid. The brain and spinal cord were removed for histological study. Serial celloidin sections at 30 and 60 μ were prepared of the brain, and paraffin sections at 15 μ of the cervical and lumbar cord, and these were stained with gallocyannin.

Histopathological Findings Typical inflammatory lesions were found at all levels of the cervical cord examined from C6 to T1. These lesions were very light, however,

The fact that spasticity can be produced without lesions being present in the spinal cord does not preclude the possibility, suggested by Kabat and Knapp,^{8,9} that lesions in the internuncial neurons of the spinal cord can produce a similar effect under certain circumstances. As Lloyd¹³ emphasizes, certain internuncial neurons in the cord are an integral part of the mechanism of reticulospinal and propriospinal action on the motoneurons. Kabat and Knapp have introduced as evidence in this connection the inconclusive observation that most spinal cords from fatal human cases have lesions in the intermediate columns where the internuncial neurons presumably are predominantly present, and that some cases have internuncial lesions with "relatively normal anterior horn cells." Unless serial sections of several segments at the involved level are examined, which was not implied, such a statement is necessarily inaccurate since lesions in the spinal cord may be quite spotty in character. For example, sections separated by only a millimeter or less may show quite different degrees of involvement of anterior, intermediate, or posterior cell columns.¹⁰ It is therefore obvious that only a quantitative study of the degree of neuron destruction in the spinal cord centers at various levels, based on a detailed study of serial sections, and correlated with symptoms referable to affected levels in each case, can yield anything more than suggestive inferences in a problem of this kind. On the other hand, the evidence from preparalytic animals, as we have noted before,¹⁸ can at times give a clear-cut answer on problems of pathogenesis because in such animals the pathological picture is in process of development and much less difficult of interpretation than that in fatal cases. The absence of lesions or detectable virus activity

in the lumbosacral cord in the preparalytic period of animals already showing spasticity of leg muscles, is an unusually good example of this.

Summary Evidence is presented which demonstrates that neither virus activity nor lesions in the spinal cord are necessary pathogenetic factors for the production of the spasticity of acute poliomyelitis. In 2 rhesus monkeys inoculated intracerebrally with poliomyelitis virus and killed in the preparalytic period when marked spasticity was present in the muscles of the legs, as well as elsewhere, no lesions were present in complete serial sections of the lumbosacral cord. In an additional similar case it was also found that no virus was present in the lumbosacral cord. In all cases severe lesions were already present in most of the brain centers usually involved, and neuronal destruction was especially severe in the midbrain tegmentum, reticular formation of the hindbrain, and in the vestibular nuclei. It is concluded that lesions in the brain alone can produce the spasticity of acute poliomyelitis. The pathological origin of this symptom is discussed, and evidence cited for the possible causative role of lesions in brain stem centers, especially the reticular formation.*

* While this paper was in press, Dr Isabel Morgan reminded the author that Bodian and Howells in 1940 (p. 152) described two relevant rhesus monkeys killed in the preparalytic stage following intracerebral virus inoculation. When killed these animals had generalized prodromal muscle tenseness, although lesions had not progressed rostrally beyond the midbrain tegmentum in one case or the hypothalamus in the other, so that no cortical lesions were found. A resurvey of serial sections of the central nervous system of both of these cases showed that lesions were absent in vestibular nuclei, very light in the cervical cord, and very severe in the reticular formation of the pons region.

¹⁸ Bodian, D., and Howe, H. A., *Brain*, 1940, 63, 135.

the results seen in Table II

Histopathological Findings Segments of cord above and below the cervical block removed for virus, of various thoracic levels, and of the upper lumbar cord were imbedded in paraffin, sectioned serially at 15 μ , stained with galloxyanin, and examined. Cord from C1 to C3 contained numerous lesions of poliomyelitis, including perivascular and diffuse leucocytic infiltration, and chromatolysis and phagocytosis of nerve cells. Sections of cord at T2, T3, T5, T9, T11, T12, L1, and L2 were quite free of any lesions of poliomyelitis.

The brain was imbedded in celloidin and sectioned at 60 μ . Every 20th section was stained with galloxyanin and examined. A typical distribution of severe focal and diffuse infiltrative lesions and of neuronal destruction was found. Neuronal destruction and inflammatory changes were particularly severe in the reticular formation, especially in the pons region, and in the vestibular nuclei. Severe changes were also present in the midbrain tegmentum, in the fields of Forel, and in the substantia nigra. Lesions in the above-mentioned centers were roughly equal on both sides, but in the forebrain there was a predominance of lesions of moderate intensity in the left dorsal thalamus and in the left pre- and postcentral cortex. Lesions in the right thalamus and cortex were very few in number, and in fact absent in most sections examined.

Summary of Experiment B430 The failure to obtain virus from the lumbar enlargement or to find lesions in the thoracic or upper lumbar cord, whereas both lesions and virus were present in the cervical cord, is sufficient evidence that virus activity had not yet reached the lumbar cord in this animal.

Discussion The evidence cited in this report indicates that neither virus activity nor lesions in the spinal cord are necessary pathogenetic factors for the production of spasticity in acute poliomyelitis. The marked spasticity seen in the legs of the monkeys described, in the absence of pathological changes in the lumbosacral cord, must be attributed to the pathological changes present in the brain or cervical region of the

spinal cord. Although the lesions in the cervical cord could have affected the long propriospinal neurons connecting with motoneurons in the lumbar cord, this is doubtful since the lesions in the cervical region were quantitatively very mild. Severe damage in the brain in primate poliomyelitis including the greatest amount of nerve cell destruction most often occurs in the reticular formation of the hindbrain and in the vestibular centers. These centers are connected with motoneurons of the spinal cord either directly or by way of internuncial neurons, as is well known.¹³ Magoun has made the interesting observation that stimulation of the bulbar reticular formation can have a generalized inhibiting effect on motor activity induced reflexly or by brain stem or cortical mechanisms,¹⁴ and Wagley¹⁵ has recently introduced evidence for the production of spasticity in rhesus monkeys by reticulospinal tract section.[†] These findings suggest that it is the severe lesions in the reticular formation in acute poliomyelitis which may be at least in part responsible for generalized spasticity, because of destruction of many "inhibitory" neurons. It is also of interest that all of the animals described in this account had almost fully developed pathological changes in the brain before limb paralysis was manifest.

¹³ Lloyd, D. P. C., *J. Neurophysiol.*, 1941, **4**, 115.

¹⁴ Magoun, H. W., *Science*, 1944, **100**, 549.

¹⁵ Wagley, P. F., *Bull. Johns Hopkins Hosp.*, 1945, **77**, 218.

[†] The cells of origin of this tract are the large neurons scattered in the central part of the reticular formation from the level of the superior colliculus caudadward.¹⁶ In a rhesus monkey prepared by Dr. Howard A. Howe and surviving 8 days after hemisection of the spinal cord at the first cervical segment, I found extensive and severe chromatolysis in these cells. The chromatolysis was bilateral but predominantly ipsilateral. It was estimated from counts made of every twentieth section of a 30 μ series, that the number of reticulospinal neurons on each side was of the order of one thousand. It is likely that these cells are part of a chain of neurons which form at least part of the extrapyramidal pathways from motor cortex to spinal cord (see review of Hines¹⁷).

¹⁶ Papez, J. W., *J. Comp. Neur.*, 1926, **41**, 365.

¹⁷ Hines, M., *Biol. Rev.*, 1943, **18**, 1.

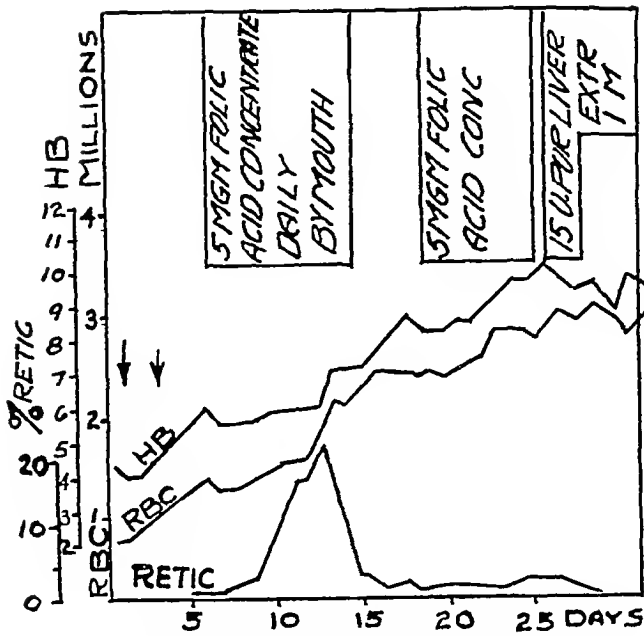


Fig 1

Case I illustrating the typical response to folic acid concentrate given orally

istic changes in the bone marrow took place as early as the fourth day. Megaloblastic erythropoiesis yielded to a normoblastic pattern and the cellularity of the femoral marrow increased markedly.

With improvement in the blood picture, the clinical symptoms disappeared, the appetite improved and healthy color returned gradually. The spleen usually remained palpable for some time. A second course of folic acid or injections of purified liver extract produced no appreciable additional response.

We consider therapy with folic acid specific in this type of anemia because (1) it produces an adequate reticulocyte response, followed by a marked, lasting improvement in the red blood counts and hemoglobin values, (2) equally important, the megaloblastic pattern of the bone marrow is transformed into a normoblastic one. Analogous changes oc-

cur in Addisonian pernicious anemia after specific therapy with liver extract^{1,2} and, recently, have been shown in nutritional macrocytic anemias of adults^{3,4}.

The genesis of this disturbance which is specifically influenced by folic acid remains obscure.

Conclusion Certain macrocytic anemias of infancy, characterized by megaloblastic erythropoiesis respond specifically to folic acid.

¹ Jones, O. P., in Downey, H., *Handbook of Hematology*, Vol. 3, p. 2045, Paul H. Hoeber, Inc., New York, 1938.

² Naegeli, O., *Wien Klin Wochensh*, 1935, **48**, 225.

³ Spies, T. D., Vilter, C. S., Koch, N. B., and Caldwell, M. H., *Southern Med J*, 1945, **38**, 707.

⁴ Moore, C. V., Vilter, R., Minnich, V., and Spies, T. D., *J. Lab. Clin. Med.*, 1944, **29**, 1226.

Folic Acid Therapy in Macrocytic Anemias of Infancy

WOLF W ZUELZER, AND F N OGDEN (Introduced by Icie G Macv)

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The clinical and hematological picture of macrocytic anemias of infancy has not been sharply defined. Favorable responses to liver extracts have been reported sporadically. In studying infants with macrocytic anemia characterized by a megaloblastic type of erythropoiesis we treated a number of cases with folic acid in the form of a concentrate prepared from liver, "L Casei factor," or with synthetic folic acid*. This report presents the results obtained with the 4 patients for whom the data have been calculated. A full report on the entire group, presenting the morphologic criteria used in determining this type of anemia, with a review of the literature, is now in preparation.

The patients were white infants, ranging from 2 to 12 months in age, one male and 3 females. All came from average homes and had been well cared for. They had received good diets, supplemented by cod liver oil and orange juice in adequate amounts. One patient (Case 3), born prematurely, had weighed 4 pounds at birth. The others had been full-term babies.

The clinical picture was not characteristic. All patients had a short history of vomiting, anorexia and pallor, as a rule preceded by signs of an upper respiratory infection and fever, which usually persisted. Three patients had had mild diarrhea, 2 had been treated with sulfadiazine.

The pertinent findings of the physical examination on admission to the hospital were marked pallor, usually slight cardiac enlargement and a soft systolic murmur. The spleen was moderately enlarged and firm.

After admission, daily complete blood counts were made. Hemoglobin was determined as oxyhemoglobin with the Evelyn photoelectric colorimeter. Reticulocyte counts were ob-

tained by the "dry" method. Bone marrow was aspirated from the lower end of the femur on admission and at intervals during the period of observation. The liquid marrow was heparinized, centrifuged and the volumetric pattern determined. Smears of the buffy coat were stained with modifications of the Romanowsky stain and differential counts of at least 500 nucleated cells were made.

All patients had severe macrocytic anemia, accompanied by moderate to marked diminution of the blood platelets. Case 2 showed mild leukopenia and neutropenia. A few nucleated red cells were invariably present in blood smears and often suggested a megaloblastic origin. The bone marrow was characterized by a megaloblastic type of erythropoiesis and changes in the granulocytes closely resembling, but not identical with, the marrow lesion in Addisonian pernicious anemia. Volumetric readings indicated moderate hypoplasia rather than hyperplasia.

In 3 cases blood transfusions were deemed necessary. These were followed by temporary increases in hemoglobin and red blood cells. Before treatment was started the hemoglobin level had begun to fall. Treatment with folic acid was not begun until preliminary observation had established the absence of a spontaneous reticulocytosis.

The response to folic acid was characterized by a rise in the reticulocyte count on the third or fourth day, which reached a maximum on the sixth or seventh day and was followed by steady rises in the hemoglobin values and red blood cell counts. The curves were virtually identical with those obtained in pernicious anemia following adequate treatment with purified liver extract (Fig 1). The hemoglobin tended to reach normal levels in approximately 3 weeks. The platelets became plentiful within one week. Character-

* Both preparations were made available through the courtesy of the Lederle Laboratories.

monochloroacetic acid, and within 2 hours almost completely abolished. The effect of di- and trichloroacetic acids, while clearly defined, is much weaker and more delayed. It is important to note that here, as at all other concentrations studied, the order of decreasing effect on respiration is monochloroacetic acid > dichloroacetic acid > trichloroacetic acid. Acetic acid in the range of 0.01M to 0.001M showed no effect.

Within the range of 0.001M to 0.03M, the depression of respiration by mono- and dichloroacetic acids is maximal; there is no indication of a progressively increasing effect with increasing concentration. Within the

range of 0.001M to 0.00005M there is a gradually decreasing effect, which ceases below 0.00005M for both acids. This is a concentration of the order of 5 to 10 μ g per milliliter of Ringer solution. At a concentration below 0.005M no effect of trichloroacetic acid has been noted, and at 0.01M evidences of tissue coagulation have been seen.

Summary At the concentrations studied monochloroacetic acid produces a marked depression in the respiration of mouse liver slices. The order of decreasing effect of the 3 acids is monochloroacetic > dichloroacetic > trichloroacetic. Acetic acid has no effect.

15265

Electrophoretic Analysis of Kala-azar Human Serum Hypergammaglobulinemia Associated with Seronegative Reactions for Syphilis*

G. R. COOPER, CHARLES R. REIN, AND J. W. BEARD

From the Department of Surgery, Duke University School of Medicine, Durham, N. C., and the Division of Serology, Army Medical School, Army Medical Center, Washington, D. C.

It is generally recognized that nonspecific positive reactions in tests for syphilis may occur with human sera from normal individuals^{1,2} and from individuals affected with certain diseases³⁻⁵ and conditions not associated with syphilis. The belief has arisen among serologists that nonspecific positive reactions are associated with hyperglobulinemia, and the presence of a high serum pro-

tein or globulin content has been suggested⁶ as a criterion of the nonspecificity of a positive reaction.

The disease, kala-azar, is characterized in part by hyperglobulinemia⁷ associated with the presence in high concentration of abnormal euglobulin, which gives rise to the formol-gel⁸ reaction and a cold-precipitable pathological globulin.⁹ In this disease, also, there has been reported¹⁰ the occurrence of nonspecific positive reaction in tests for syphilis. Recently, in connection with a comparative study

* The work described in this paper was done under contract, recommended by the Committee on Medical Research, between the Office of Scientific Research and Development and Duke University.

¹ Eagle, H., *Am J Syph*, 1941, **25**, 7.

² Mohr, C. F., Moore, J. E., and Eagle, H., *Arch Int Med*, 1941, **68**, 898.

³ Mohr, C. F., Moore, J. E., and Eagle, H., *Arch Int Med*, 1941, **68**, 1161.

⁴ Hazen, H. H., Parran, T., Sanford, A. H., Sencer, F. E., Simpson, W. M., and Vonderlehr, R. A., *Internat J Leprosy*, 1936, **4**, 315.

⁵ Davis, B. D., *Medicine*, 1944, **23**, 359.

⁶ Cirdon, L., and Atlas, D. H., with the assistance of Aron, E., Brunner, M. J., Teitelman, S. L., and Bunzli, J., *Arch Dermat and Syph*, 1942, **46**, 713.

⁷ Lloyd, R. B., and Paul, S. N., *Indian J Med Res*, 1928 1929, **16**, 203.

⁸ Napier, L. E., *Indian J Med Res*, 1921 1922, **9**, 830.

⁹ Wertheimer, E., and Stein, L., *J Lab and Clin Med*, 1944, **29**, 1082.

¹⁰ Greil, S. D. S., Gupta, P. C. S., and Napier, L. E., *Indian J Med Res*, 1939 1940, **27**, 181.

Effect of Acetic, Monochloroacetic, Dichloroacetic, and Trichloroacetic Acids on Oxygen Consumption of Mouse Liver

EDWIN P LAUG (Introduced by O Garth Fitzhugh)

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From acute oral toxicity data in mice, rats and guinea pigs¹ it has been shown that monochloroacetic acid is from 25 to 40 times as toxic as acetic acid and its di- and trichloro derivatives. It has not been determined from these studies what causes death nor have chronic feeding experiments indicated any clearly defined functional or organic derangements. While the effect of monoiodoacetic acid on the glycolytic and oxidative functions of tissues has been amply demonstrated, such information on monochloroacetic acid is at best somewhat meager. It has been recorded that monochloroacetic acid has chologogic action² and Lundsgaard³ states that in comparison to monoiodoacetic acid, monochloroacetic acid inhibits yeast fermentation only feebly. The present experiments were

undertaken to determine what effect, if any, mono-, di- and trichloroacetic acid would have on the respiration of liver tissue.

Method Liver slices from well fed young adult mice were used. Oxygen consumption was measured by the standard Warburg procedure at 37.5°C. Acetic, mono-, di-, and trichloroacetic acids were neutralized and diluted in phosphate buffered glucose-Ringer, so that the final concentration in contact with the liver slice ranged from 0.03M to 0.00005M. The pH of all solutions was adjusted to 7.4.

Results Fig 1 shows in graphic form the typical effects of mono-, di-, and trichloroacetic acids at 0.0005M concentration on the respiration of mouse liver slices. Respiration is promptly and precipitously reduced by

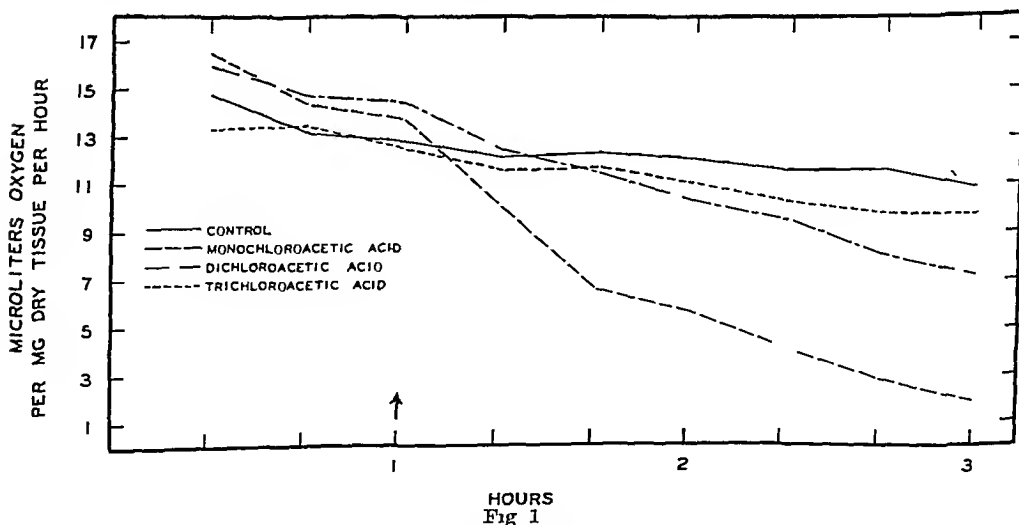


Fig 1
Effect of 0.0005M mono-, di-, and trichloroacetic acids on oxygen consumption of mouse liver slices. Temp 37.5°C, pH 7.4

¹ Woodward, G, Lange, S W, Nelson, K W, and Calvery, H O, *J Indust Hyg and Tox*, 1941, 23, 78

² Chabrol, E, Charonnat, R, Maximin, M, and Wartz, R, *Compt rend Soc Biol*, 1931, 106, 17

³ Lundsgaard, E, *Biochem Z*, 1932, 250, 61

monochloroacetic acid, and within 2 hours almost completely abolished. The effect of di- and trichloroacetic acids, while clearly defined, is much weaker and more delayed. It is important to note that here, as at all other concentrations studied, the order of decreasing effect on respiration is monochloroacetic acid > dichloroacetic acid > trichloroacetic acid. Acetic acid in the range of 0.01M to 0.001M showed no effect.

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⁴ Hazen, H. H., Parran, T., Sanford, A. H., Sencer, F. E., Simpson, W. M., and Vonderlehr, R. A., *Internat J Leprosy*, 1936, **4**, 315.

⁵ Davis, B. D., *Medicine*, 1944, **23**, 359.

⁶ Curdon, L., and Atlas, D. H., with the assistance of Aron, E., Brunner, M. J., Teitelman, S. L., and Bunati, J., *Arch Dermat and Syph*, 1942, **46**, 713.

⁷ Lloyd, R. B., and Paul, S. N., *Indian J Med Res*, 1928-1929, **16**, 203.

⁸ Napier, L. E., *Indian J Med Res*, 1921-1922, **9**, 830.

⁹ Wertheimer, E., and Stein, L., *J Lab and Clin Med*, 1944, **29**, 1082.

¹⁰ Grevil, S. D. S., Gupta, P. C. S., and Napier, L. E., *Indian J Med Res*, 1939-1940, **27**, 181.

TABLE I
Percentage Distribution, Concentration and Mobilities of the Components of
Normal and Kala azar Human Sera

	Sample	Serum	Electrophoresis Solution	Components				
				Albumin A	Globulins			
					α_1	α_2	β	γ
%	477			21.7	3.0	3.4	7.8	64.1
Distribution	499			50.3	2.3	11.6	7.4	28.4
	Normal*			60.0	3.6	9.1	12.5	14.8
Protein	477	12.1	2.88	2.62	0.36	0.41	0.95	7.76
Concentration	499	8.65	2.58	4.35	0.20	1.00	0.64	2.46
gm per 100 ml	Normal*	7.23	—	4.34	0.26	0.66	0.90	1.07
Mobility	477			5.88	5.00	3.94	2.79	0.58
$-\mu \times 10^5$	499			5.98	5.04	3.91	3.08	0.62
cm ² sec ⁻¹ volt ⁻¹	Normal*			6.08	5.04	3.93	2.97	1.27

* Avg values of 13 normal human sera¹¹

of syphilitic and nonspecific positive sera,¹¹ an opportunity was afforded for the examination of a serum from each of 2 patients with kala-azar. Electrophoretic analyses were made of the distribution, concentration and mobilities of the serum protein components, and the sera were thoroughly studied with a battery of serodiagnostic tests for syphilis. The results of these electrophoretic and serological studies are reported in the present paper.

Materials and Methods The sera† were obtained from 2 patients in whom the diagnosis of kala-azar was established by the recovery of Leishman-Donovan bodies by puncture of the spleen and sternal marrow. One, Serum 477, was from a 23-year-old colored male, C W Y, who became ill February 28, 1944. The serum proteins were consistently elevated to levels reaching 12 g per 100 ml. The blood for electrophoretic analysis was drawn September 8, 1944, while the patient was still clinically ill. The second patient, Serum 499, was a 24-year-old white male, A W F, who had malaria in the summer of 1943 and typhoid in January, 1944. The onset of kala-azar occurred in April, 1944.

The total serum protein and globulin contents were 6.7 and 3.35, 8.9 and 4.3, and 7.75 and 2.75 gm per 100 ml in July, August and October, respectively. The serum for the present studies was drawn on November 17, 1944, about 30 days after abatement of the disease.

Electrophoresis was carried out as previously described^{11,12} at 1° in barbital buffer¹³ at pH 8.6 and 0.1 ionic strength. The period of migration of Serum 477 was 234 minutes at 5.95 volts per cm and of Serum 499, 180 minutes at 6.42 volts per cm. The patterns representing the migrating boundaries were recorded by the method of crossed slits described by Svensson.¹⁴

The serum was prepared for electrophoresis by dilution with the barbital buffer to approximately 2% protein content and dialysis against frequently changed buffer at 2 to 8°. Protein concentrations were determined by the falling-drop procedure¹⁵ standardized by Kjeldahl analyses.

Component analyses were made from measurements in photographic enlargements of the electrophoretic diagrams in which the components were separated by vertical lines.

¹¹ Cooper, G. R., Craig, H. W., and Beard, J. W., in press.

† The sera and histories of the patients were provided through the generosity of Capt James Liebmman, M. C., A. U. S., Walter Reed General Hospital, Washington, D. C.

¹² Sharp, D. G., Taylor, A. R., Beard, D., and Beard, J. W., *J. Biol. Chem.*, 1942, **142**, 193.

¹³ Longworth, L. G., *Chem. Rev.*, 1942, **30**, 323.

¹⁴ Svensson, H., *Kolloid Z.*, 1940, **90**, 141.

¹⁵ Barbour, H. G., and Hamilton, W. F., *J. Biol. Chem.*, 1926, **69**, 625.

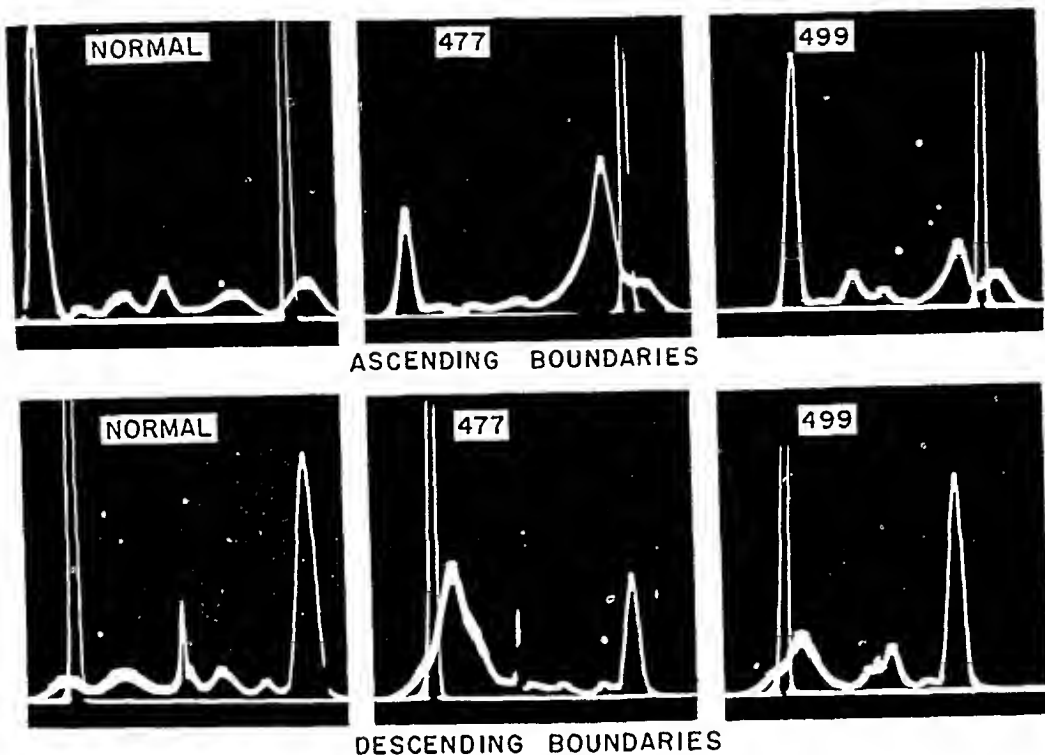


Fig 1

Electrophoretic patterns of human serum from a normal individual and from 2 patients diseased with kala azar

to the base at the minima between the peaks¹⁰. The respective areas were bounded by the mid-line of the curve above and the mid-line of the base below. The mobilities of the various components were calculated from the distances measured from the starting point of migration to the maximum ordinates of the respective peaks¹³. Calculations were made for both ascending and descending sides and the values given in Table I are the average of the 2 sets of data.

The sera of both patients were subjected to serologic examinations with a battery of 6 serodiagnostic tests, including the Kline diagnostic, Kline exclusion, Boerner-Jones-Lukens, Mazzini, Kahn and Kolmer procedures. The tests were made on other sera drawn in August, September and October of 1944 in addition to the 2 sera on which the electrophoretic studies were made.

Further, the blood specimen, Serum 477, drawn from patient C W F on September 8, 1944, was also subjected to chemical fractionation by Dr Hans Neurath, Duke University School of Medicine, and each fraction, the albumin and the GI, GII and GIII globulins, was tested with the 5 flocculation procedures enumerated above. Formol-gel tests⁸ were made on both of the sera.

Results Electrophoretic patterns of the ascending and descending boundaries of the kala-azar sera and of a normal¹¹ serum for comparison are reproduced in Fig 1. The pattern of Serum 477 reveals a γ globulin peak much greater in height and area than the albumin peak. In addition, there is pronounced skewing of the γ globulin peak to the slow side and diffuseness of the curve on the fast side. The percentage distribution and actual concentration of the components of Serum 477 are given in Table I. It is seen that the γ globulin fraction was

¹⁰ Tiselius, A., and Kibrit, E. A., *J. Exp. Med.*, 1930, 69, 119.

about 7 times the normal value,¹¹ while the albumin was less than one-half the normal. The other electrophoretic fractions showed insignificant deviation from the concentrations observed in normal serum.

The mobilities of the proteins of Serum 477 (Table I) were the same as those of the components of normal serum,¹¹ except in the instance of the γ globulin fraction. The mobility of this fraction was outside the limits of the mobility usually associated with normal human γ globulin.¹¹ The diffuseness in the curve in the early part of the γ peak indicates the presence of the normal γ component.

Similar abnormalities but of less degree were observed in the proteins of Serum 499. The γ globulin peak was sharply heightened and skewed to the slow side of the γ globulin region. There was little suggestion of disturbance of the curve corresponding to the region of the normally behaving γ globulin. In percentage composition (Table I) the γ globulin fraction was double the normal, and the albumin and β globulin fractions were slightly less than normal. The actual concentrations of the components (Table I) showed that no significant changes from the normal had occurred except in the γ globulin fraction. The β anomaly peak¹³ was absent. Mobilities of the components were within the normal range except that of the γ globulin fraction, which was found to be the same as in Serum 477.

The serodiagnostic tests on all of the serum samples investigated were uniformly negative and so, too, were the tests of the albumin and GI, GII and GIII globulins obtained by chemical fractionation. The sera, likewise, were negative to the formol-gel test.

Discussion The abnormalities of the γ globulin component of kala-azar sera differ greatly from those of the serum of patients with other diseases involving the liver and spleen.^{17,18} In syphilis,¹¹ rheumatic fever,¹⁹

sarcoidosis,^{20,21} tuberculosis,²² as well as other pathological states,²³⁻²⁵ the rise in γ globulin is associated with the entire normal γ globulin region. The findings indicate the presence in human kala-azar serum of a γ globulin component not previously observed in human serum. A γ globulin²⁶ component of low mobility has been observed, however, in the serum of rabbits hyperimmunized with Type 1 pneumococci.

The possible relation between the abnormal γ globulin and the globulins giving the formol-gel reaction⁸ or precipitating in the cold⁹ is subject only to surmise. Since the formol-gel test was negative, the abnormal γ globulin could not have been representative of the whole of the material responsible for this reaction. The cold-precipitable euglobulin, which may persist after recovery, may have contributed to the γ globulin. The electrophoretic abnormalities, pronounced during the course of the disease and still present in the convalescent serum, may prove on further investigation to be unique with kala-azar and of greater diagnostic significance than the formol-gel test or cold precipitation.

The nonspecific positive reactions in tests for syphilis seen in a variety of nonsyphilitic diseases and conditions may be due to the presence of antibody-like substances similar to the antibodies produced in syphilitic disease, to an increase or alteration of some chemical substance or substances in the blood, or to an increase or alteration of the seroglobulin fraction. Cardon and Atlas⁶ investigated a series of 34 patients with hyperproteinemia, of which 8 gave nonspecific positive reactions for syphilis. These au-

²⁰ Seibert, F. B., and Nelson, J. W., *Am Rev Tuberc*, 1943, **47**, 66.

²¹ Fisher, A. M., and Davis, B. D., *Bull Johns Hopkins Hosp*, 1942, **71**, 364.

²² Seibert, F. B., and Nelson, J. W., *J Biol Chem*, 1942, **143**, 29.

²³ Longworth, L. G., Shedlovsky, T., and MacInnes, D. A., *J Exp Med*, 1939, **70**, 399.

²⁴ Luetscher, J. A., Jr., *J Clin Invest*, 1941, **20**, 99.

²⁵ Shedlovsky, T., and Seudder, J., *J Exp Med*, 1942, **75**, 119.

²⁶ van der Seheer, J., Bohnel, E., Clarke, F. H., and Wyckoff, R. W. G., *J Immunol*, 1942, **44**, 165.

¹⁷ Luetscher, J. A., Jr., *J Clin Invest*, 1940, **19**, 313.

¹⁸ Gray, S. J., and Brannon, E. S. G., *J Clin Invest*, 1943, **22**, 191.

¹⁹ Rutstein, D. D., Clarke, F. H., and Taran, L. M., *Science*, 1945, **101**, 669.

thors believe that blood protein studies should be instituted when a nonspecific positive reaction is suspected and that care should be taken in the interpretation of a positive serologic reaction for syphilis in the presence of conditions with hyperproteinemia and hyperglobulinemia. Proven syphilitics may also show¹¹ a hypergammaglobulinemia and patients with kala-azar, as shown in the present work, and sarcoidosis²⁷ may have hyperglobulinemia associated with negative serologic reactions for syphilis. The mere presence or

absence of hyperproteinemia, hyperglobulinemia or hypergammaglobulinemia is not sufficient to prove or disprove the specificity of positive serologic reactions for syphilis.

Summary The electrophoretic patterns of 2 human kala-azar sera revealed the presence in high concentration of a unique, abnormal component migrating with the γ globulin of the slowest mobility. The kala-azar sera, negative to serological tests for syphilis, provide instances which show that the presence of hyperproteinemia, hyperglobulinemia or hypergammaglobulinemia cannot be used to prove or disprove the specificity of positive serologic reactions for syphilis.

²⁷ Cooper, G. R., Rem, C. R., and Bruid, J. W., unpublished observations.

15266

Inactivation of Glutamic-Aspartic Transaminase by Irradiation*

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Glutamic-aspartic transaminase is an enzyme which catalyzes the reaction $1 (+)$ glutamic acid + oxaloacetic acid \rightleftharpoons α -ketoglutaric acid + $1 (-)$ aspartic acid. The prosthetic group of this enzyme is a derivative of vitamin B₆,^{1,2} probably identical with codecarboxylase.^{3,4} Since pyridoxine, pyridoxal and pyridoxamine are very sensitive to light,^{5,6,7}

it was expected that transaminase preparations would also be inactivated by irradiation.

It was found that sunlight destroys the enzymatic activity at about the same rate as it inactivates the vitamin B₆ present in the preparations (Table I). The destruction by ultraviolet light is much more rapid (Table II). The effect of X-rays on transaminase was also investigated. Vitamin B₆ has been considered as a remedy for X-ray sickness^{8,9}. It was found that X-ray irradiation in therapeutic and higher doses has no effect on transaminase activity. Therefore, the possible therapeutic value of vitamin B₆ in this condition cannot be explained in terms of a restoration of inactivated transaminase.

Experimental Procedures For the determination of transaminase activity the procedures developed by Cohen¹⁰ were used. The enzyme was purified according to the

* Published with the approval of the Director of the Wisconsin Agricultural Experiment Station. Reported at the Dills Meeting, Southwestern Section, on October 20, 1945.

¹ Schlenk, F., and Snell, E. E., *J. Biol. Chem.*, 1945, **157**, 425.

² Schlenk, F., and Fisher, A., *Arch. Biochem.*, 1945, **8**, 337.

³ Lichstein, H. C., Gunsalus, I. C., and Umbreit, W. W., *J. Biol. Chem.*, 1945, **161**, 311.

⁴ Green, D. E., Leloir, L. F., and Noerto, V., *J. Biol. Chem.*, 1945, **161**, 559.

⁵ Hochberg, M., Melnick, D., Siegel, L., and Oser, B. L., *J. Biol. Chem.*, 1943, **148**, 253.

⁶ Hochberg, M., Melnick, D., and Oser, B. L., *J. Biol. Chem.*, 1944, **155**, 129.

⁷ Cunningham, D., and Snell, E. E., *J. Biol. Chem.*, 1945, **158**, 491.

⁸ Maxfield, J. R. Jr., McIlwain, A. J., and Robertson, J. E., *Radiology*, 1943, **41**, 383.

⁹ Gendreau, J. E., and Lafleur, L., *Union med. du Canada*, 1944, **73**, 666.

¹⁰ Cohen, P. P., *J. Biol. Chem.*, 1940, **136**, 565.

TABLE I
 Inactivation of Glutamic Aspartic Transaminase by Sunlight

Time (hrs)	Irradiated		Dark controls			
	Vitamin B ₆ present is determined with*		Vitamin B ₆ present is determined with*			
	Transaminase activity %	<i>L. casei</i> γ per g	<i>S. fecalis</i> R γ per g	<i>S. cerevisiae</i> γ per g	<i>S. cerevisiae</i> R γ per g	<i>S. cerevisiae</i> γ per g
0	30.5	67	30.5	67	30.5	95
1	14.9	24	40	47	267	75
2	(1.9)	19	39	56	276	83
3	6.1	11	22	50	305	79

* Pyridoxal was used as standard with *L. casei*, pyridoxamine with *S. fecalis* R, and pyridoxine with *S. cerevisiae*

method outlined earlier² which yields solutions with no absorption in the visible part of the spectrum

Irradiation by Sunlight For irradiation by sunlight large open Petri dishes were placed in an icebath. The light path was 0.4 cm, irradiation was from 1 P.M. to 4 P.M., May 14, 1945, 29° North latitude. At the times given in Table I samples were withdrawn for the determination of transaminase and for the microbiological assay of the B₆ vitamins.¹¹

Microbiological assay was carried out following 15 minutes autoclaving of the sample with N/10 sulfuric acid. No attempt at differential assay¹² for various members of the vitamin B₆ group was made, since the relative activity of the coenzyme for the various organisms and the conditions which effect its complete hydrolysis and release from the protein molecule with which it is associated are at present unknown.⁴ The figures given in Table I represent the total vitamin B₆ per gram of protein as indicated by assay with each organism against the appropriate standard.

Irradiation by Ultraviolet Light For ultraviolet irradiation a quartz mercury lamp (General Electric Co., Model F, 400 watt) was used. The distance between the lamp and the solution was 45 cm. Otherwise the procedure outlined above was followed. For comparison solutions of pyridoxine, pyridoxal, and pyridoxamine (10 γ per ml) were irradiated under the same conditions. The destruction was determined using the colorimetric reaction of Swaminathan¹³ in the modification of Bina, Thomas, and Brown.¹⁴

The inactivation as recorded in Tables I and II is not due to hydrogen peroxide. The hydrogen peroxide concentration in distilled water irradiated by ultraviolet light under the same conditions for 30 minutes remained below 6×10^{-5} moles per liter. We found

¹¹ Snell, E. E., and Rønnefeld, A. N., *J. Biol. Chem.*, 1945, **157**, 475.

¹² Snell, E. E., *J. Biol. Chem.*, 1945, **157**, 491.

¹³ Swaminathan, M., *Nature (London)*, 1940, **145**, 780.

¹⁴ Bina, A. F., Thomas, J. M., and Brown, E. B., *J. Biol. Chem.*, 1943, **148**, 111.

TABLE II
 Inactivation of Transaminase and of Vitamin B₆ by Ultraviolet Light

Irradiation (min)	Transaminase activity %	Vitamin B ₆ compounds		
		Pyridoxal γ	Pyridoxamine γ	Pyridoxine γ
0	37.2	40.0	40.0	40.0
5	10.3	8.0	18.5	14.3
10	4.3	2.1	9.8	8.9
15	1.9	1.2	5.9	4.3
30	<1	0.6	2.9	0.8

that transaminase is rather resistant to hydrogen peroxide. The stability of the members of the vitamin B₆ group has been reported earlier.⁷ The inactivated enzyme was not reactivated by addition of pyridoxine, pyridoxal, pyridoxamine, or boiled tissue extract.

Irradiation by X-rays The conditions were as follows: 200 K V, 16 milliamperes, 50 cm distance, filter, 2.5 mm Al inherent in tube, none added. Since no appreciable inactivation of transaminase (Table III) was observed, no vitamin B₆ determinations were carried out.

Summary Glutamic-aspartic transaminase

† The authors are indebted to Dr. T. G. Russell for collaboration.

 TABLE III
 X-Ray Irradiation of Transaminase

Irradiation 1 units	Transaminase activity %
0	39.7
500	36.2
1,500	38.4
4,000	35.8
7,000	39.1
10,000	34.5

is inactivated by sunlight and by ultraviolet light. Destruction of enzymatic activity by these agents parallels the destruction of vitamin B₆, a derivative of which is present as the prosthetic group of the enzyme.¹⁻⁴ The irradiated enzyme is not reactivated by addition of pyridoxine, pyridoxal, pyridoxamine or boiled tissue extracts. Irradiation with X-rays leaves the enzyme intact.

15267

Biological Activity and Metabolism of d,l-O-Heterobiotin in the Chick

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O-Heterobiotin, an oxygen analog of biotin, has biotin-like activity for the chick¹ and for rats fed egg white.^{1,2} Hofmann *et al.*¹ found the d,l analog 10% as active as d-biotin in curing egg-white toxicity in rats and approximately 6 and 20% as active in promoting

growth in chicks when fed at different levels on a biotin deficient ration. Rubin *et al.*² found d,l-O-heterobiotin 5% as active as d-biotin in counteracting egg-white toxicity in the rat.

Since levels of 40 γ of d,l-O-heterobiotin

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We are indebted to Merck and Company, Rah-

way, N. J., for crystalline vitamins, to Dr. S. H. Rubin and Dr. J. A. Aeschlimann of Hoffmann-La Roche, Inc., Nutley, N. J. for O-heterobiotin, to Dr. B. L. Hutchings of Lederle Laboratories, Inc., for crystalline folic acid, to Abbott Laboratories, North Chicago, for haliver oil, to E. I.

TABLE I
Activity and Storage of d,l O Heterobiotin* in the Chick

Group No	Supplement per 100 g biotin deficient basal	No of survivors at 4 wks	Avg wt at 4 wks	No of chicks with dermatitis at 4 wks	d,l O Heterobiotin content of	
					Liver	Leg muscle
1	None	11†	g 185	11	γ/g 0 0	γ/g 0 0
2	25 γ d biotin	6	185	3		
3	5 γ "	3	205	0		
4	10 γ "	5	220	0		
5	20 γ "	11†	240	0	0 0	0 0
6	5 γ d,l O heterobiotin	6	180	4		
7	10 γ "	6	175	2		
8	20 γ "	6	195	0	0 3	0 0
9	200 γ "	6	205	0		
10	500 γ "	4	215	0	0 5	0 0
11	1000 γ "	6	210	0	5 3	0 2

* d,l O Heterobiotin is compared with d biotin on a weight basis throughout this work. If only the d form of the analog is active the values should be increased correspondingly.
† 12 chicks per group

per 100 g of a biotin-deficient ration have given only partial responses in chicks¹ it was interesting to determine if higher levels could eventually give complete protection. At the same time it was possible to study the distribution of the analog in chick tissues when graded levels of the compound were included in the diet.

Day-old White Leghorn cockerels were maintained in cages with raised screen bottoms and heated by carbon filament lamps. The chicks were fed a purified ration having the following composition: dextrin 61 g, casein 18 g, gelatin 10 g, Salts V³ 6 g, soy bean oil 5 g, 1(—) cystine 0.3 g, L-inositol 0.1 g, choline chloride 0.15 g, thiamine hydrochloride 0.3 mg, pyridoxine hydrochloride 0.4 mg, riboflavin 0.6 mg, Ca pantothenate 2 mg, nicotinic acid 5 mg, 2-methyl-1,4-naphthoquinone 0.05 mg, α-tocopherol 0.3 mg, p-aminobenzoic acid 5 mg, and crystalline folic acid 0.05 mg. Water and feed were

given *ad libitum*, and each chick received weekly one drop of a vitamin A and D concentrate containing 1200 USP units of vitamin A and 120 AOAC units of crystalline vitamin D₃ per drop. After 3 days on the basal ration chicks within a 10 g weight range were divided into uniform groups of 6 and given supplements of d-biotin or d,l-O-heterobiotin in the basal ration. The feeding trials were terminated when the chicks were 4 weeks old and samples of liver and leg muscle were taken from 4 chicks in each group for O-heterobiotin analysis. O-Heterobiotin was determined by the differential microbiological assay of Luckey *et al*.⁴

The average weight at 4 weeks, incidence of dermatitis, and O-heterobiotin content of the tissues are summarized in Table I. A comparison of the relative effectiveness of d,l-O-heterobiotin and d-biotin in curing dermatitis indicates that the d,l analog is about 1/3 as active as d-biotin. However, in a comparison based on growth the activities varied inversely with the amount of analog fed. 20 γ of the d,l analog had an activity of approximately 20%. With higher levels the activity fell off rapidly and values from

du Pont de Nemours and Company, Inc., New Brunswick, N. J., for crystalline vitamin D₃, to Wilson and Company, Inc., Chicago, for gelatin, to Allied Mills, Inc., Peoria, Ill., for soy bean oil, and to Mr. J. Kimlin for technical assistance.

¹ Hofmann, K., McCoy, R. H., Felton, J. R., Axelrod, A. E., and Pilgrim, F. J., *Arch Biochem*, 1945, 7, 393.

² Rubin, S. H., Flower, D., Rosen, F., and Drexler, L., *Arch Biochem*, 1945, 8, 79.

³ Briggs, G. M., Luckey, T. D., Elvehjem, C. A., and Hart, D. B., *J Biol Chem*, 1943, 148, 163.

⁴ Luckey, T. D., Moore, P. R., and Elvehjem, C. A., in press.

0.5 to 3% were obtained. It is apparent that a level of d,l-O-heterobiotin as high as 1000 γ per 100 g of ration does not give a complete growth response in chicks on our biotin deficient ration and it appears that higher levels would not have afforded complete protection since the 1000 γ level actually gave no better response than the 500 γ level. This failure of O-heterobiotin to completely replace d-biotin for growth when it is highly active in the prevention of dermatitis indicates that the analog is capable of fulfilling only part of the function of d-biotin in the chick. On the other hand, the plateau in growth observed above the 500 γ level (compare group 11 to 10) suggests an inhibitory effect of the d,l analog on growth. In this connection, Luckey *et al.*⁴ have observed an inhibitory effect of low levels of d,l-O-heterobiotin on the growth of *Streptococcus fecalis*. Further experimental data are required to clarify this point.

The analyses for d,l-O-heterobiotin indicate

that this compound does not occur naturally in chicks receiving our biotin deficient diet alone or when supplemented with d-biotin. No appreciable accumulation of the d,l analog occurred in the livers of the chicks receiving O-heterobiotin until 1000 γ were included in the diet. It is interesting to note that this represents the same level at which a growth plateau was established. Similarly, no O-heterobiotin could be detected in muscle tissue until the 1000 γ level of the analog was reached.

Summary. d,l-O-Heterobiotin has biotin-like activity for the chick. Levels of 20 γ per 100 g of purified biotin-deficient basal ration give complete protection against dermatitis and some growth response. The growth activity of the oxygen analog varies inversely with the level fed and growth comparable to that on an adequate biotin diet was not obtained. d,l-O-Heterobiotin can be detected in the liver and muscle tissue of chicks fed high amounts of this compound.

15268

The Inactivation of Streptomycin and its Practical Applications *†

WALTON B. GEIGER, SAMUEL R. GREEN, AND SELMAN A. WAKSMAN

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Because of the rapidly accumulating information on the production, purification and practical utilization of streptomycin,¹ a knowledge of the effects of experimental conditions upon its activity is increasingly important. It is known, for example, that a weakly alkaline environment, namely pH 8.0 to 8.2, is favorable to the action of streptomycin and that acidic conditions are injurious.² Likewise, its activity is diminished

by the presence of glucose and of phosphates. Cysteine and several ketone reagents also inactivate streptomycin.³ Since factors of this sort can be of importance in choosing media for the testing of the potency of streptomycin, in developing tests for the sterility of streptomycin preparations, and in other practical applications, it was believed desirable to re-investigate and extend some of the earlier observations. The presence of reducing agents, such as cysteine and glucose, among the substances that inactivate streptomycin, as well as the observation that anaerobic bacteria are somewhat resistant to its activity, has suggested that special attention be paid to these conditions, in an effort to learn whether their effects are of a fundamental

* Journal Series Paper, New Jersey Agricultural Experiment Station, Rutgers University. Department of Microbiology.

† Partially supported by a grant made by the Commonwealth Fund of New York.

¹ Waksman, S. A. and Schutz, A. J. *Am. Pharm. Assoc.* 1945, **34**, 273.

² Waksman, S. A., Bugie, E., and Schutz, A. J. *Proc. Staff Meet. Mayo Clinic*, 1944, **19**, 537.

³ Denkewalter, R., Cook, M., and Tishler, M. *Science*, 1945, **101**, 12.

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6	5 γ d,l O heterobiotin	6	180	4		
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per 100 g of a biotin-deficient ration have given only partial responses in chicks¹ it was interesting to determine if higher levels could eventually give complete protection. At the same time it was possible to study the distribution of the analog in chick tissues when graded levels of the compound were included in the diet.

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given *ad libitum*, and each chick received weekly one drop of a vitamin A and D concentrate containing 1200 USP units of vitamin A and 120 AOAC units of crystalline vitamin D₃ per drop. After 3 days on the basal ration chicks within a 10 g weight range were divided into uniform groups of 6 and given supplements of d-biotin or d,l-O-heterobiotin in the basal ration. The feeding trials were terminated when the chicks were 4 weeks old and samples of liver and leg muscle were taken from 4 chicks in each group for O-heterobiotin analysis. O-Heterobiotin was determined by the differential microbiological assay of Luckey *et al*.⁴

The average weight at 4 weeks, incidence of dermatitis, and O-heterobiotin content of the tissues are summarized in Table I. A comparison of the relative effectiveness of d,l-O-heterobiotin and d-biotin in curing dermatitis indicates that the d,l analog is about 1/3 as active as d-biotin. However, in a comparison based on growth the activities varied inversely with the amount of analog fed. 20 γ of the d,l analog had an activity of approximately 20%. With higher levels the activity fell off rapidly and values from

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¹ Hofmann, K., McCoy, R. H., Felton, J. R., Axelrod, A. E., and Pilgrim, F. J., *Arch. Biochem.*, 1945, **7**, 393.

² Rubin, S. H., Flower, D., Rosen, F., and Drexler, L., *Arch. Biochem.*, 1945, **8**, 79.

³ Briggs, G. M., Luckey, T. D., Elvehjem, C. A., and Hurl, E. B., *J. Biol. Chem.*, 1943, **148**, 163.

⁴ Luckey, T. D., Moore, P. R., and Elvehjem, C. A., in press.

TABLE I
Effect of Atmosphere Upon Action of Streptomycin

Atmosphere	Activity in dilution units per ml against			
	<i>E. coli</i>	<i>A. aerogenes</i>	<i>P. vulgaris</i>	<i>S. aureus</i>
Air	500	500	500	500
H ₂	200	400	100	20
N ₂	100	100	100	80
CO ₂	20	20	30	20

TABLE II
Effect of Bicarbonate and Acidity Upon Action of Streptomycin

	Activity in units/ml against			
	<i>E. coli</i>	<i>A. aerogenes</i>	<i>S. aureus</i>	<i>B. subtilis</i>
Nutrient agar (pH 6.8)	100	100	100	1 000
Nutrient agar (pH 5.7)	10	10	5	100
Nutrient agar + 2% NaHCO ₃	100	100	100	800

nature or are due to secondary factors such as fall in pH value

Streptomycin is a rather stable compound. No biological agent or enzyme system has yet been found capable of destroying it. At 25°C, it is not affected appreciably between pH 2.0 and 9.0, but it is decomposed by excessive acidity (1 *N* HCl) and alkalinity (0.1 *N* NaOH). Hydrolysis by hydrochloric acid in methanol has been shown to result in the splitting of the molecule with the liberation of a base, streptidine, and a carbohydrate-like substance, streptobiosamine, neither of which possesses much, if any, antibiotic activity. The streptidine is believed to be attached to the streptobiosamine by a glucosidic linkage.⁴

Aerobic vs Anaerobic Environment In order to learn whether the greater resistance of anaerobes than of aerobes to streptomycin was due to the nature of the organisms or to the environment, a study was first made of the activity of streptomycin on several bacteria growing in different environments. Organisms that are facultative anaerobes, *i.e.*, that can grow either in air or with a limited supply of oxygen, were selected. A highly active streptomycin preparation containing 500 units/ml was used. Serial dilutions of

the streptomycin solution were made in nutrient agar, and 4 series of plates were made up with this streptomycin-containing agar and streaked with the 4 test bacteria. One series of plates was incubated at 28°C under the usual aerobic conditions, and the other 3 series were incubated under anaerobic conditions in atmospheres of hydrogen, nitrogen, and carbon dioxide. The results presented in Table I show that streptomycin is about one-half as active under hydrogen as in air, considerably less active under nitrogen, and almost completely inactive under carbon dioxide.

Two supplementary tests were made in an effort to elucidate the effect of carbon dioxide. First, it was determined that the pH of nutrient agar was changed from 6.8 to 5.7 by storage for 16 hours under carbon dioxide. Tests showed that on agar adjusted to this pH, streptomycin had its activity diminished to about the same degree as by carbon dioxide. The effect of streptomycin was now tested on nutrient agar to which sodium bicarbonate had been added (Table II). No inactivation of streptomycin took place. One may, therefore, conclude that the effect of carbon dioxide is probably a result of the change in pH produced by it in the medium.

In order to determine whether the di-

⁴ Brink, N. G., Kuehl, J. I., F. A., and Folkers, K., *Science*, 1945, 102, 506.

TABLE III
Changes in pH Value Produced by 4 Test Bacteria
in Different Environments

Atmosphere	Organism	pH*
Air	<i>E. coli</i>	7.50
	<i>A. aerogenes</i>	7.80
	<i>Sci. marcescens</i>	7.35
	<i>Pr. vulgaris</i>	7.13
Nitrogen	<i>E. coli</i>	7.25
	<i>A. aerogenes</i>	7.54
	<i>Sci. marcescens</i>	6.68
	<i>Pr. vulgaris</i>	6.69
Hydrogen	<i>E. coli</i>	6.63
	<i>A. aerogenes</i>	6.84
	<i>Sci. marcescens</i>	6.48
	<i>Pr. vulgaris</i>	6.43

* Original pH 7.0

TABLE IV
Effect of Sugars Upon Susceptibility of *B. subtilis*
to Streptomycin

Sugar added*	pH after growth	Cup method zone size in mm
None	7.1	19.3
Dextrose	6.3	10.1
Levulose	6.3	10.0
Sucrose	5.3	10.4
Mannose	7.1	18.4
Galactose	7.1	17.8
Arabinose	6.9	14.2
Maltose	7.0	18.5
Lactose	7.1	17.2
Mannitol	6.7	10.0

* 1% in nutrient agar

diminished activity of streptomycin in nitrogen or hydrogen could be a result of the production of acid by the test organisms under these conditions, a study was made of the changes in pH of the cultures. Flasks of nutrient broth were inoculated with the above 4 cultures and kept under air, hydrogen, and nitrogen. After incubation for 24 hours, pH determinations were made (Table III). The results suggest that the production of acid by the test bacteria under anaerobic conditions may be at least one factor in the diminished activity of streptomycin under these conditions.

Effect of Sugars The action of streptomycin on *B. subtilis* is known to be diminished by glucose.¹ In order to obtain further information concerning this effect, the following 2 experiments were conducted. Since glucose is fermented by *B. subtilis* with the formation

of acid, and since streptomycin is less active at low pH, it seemed likely that the action of glucose was largely a pH effect. A study was therefore made of the production of acid by *B. subtilis* from several sugars, as well as the effect of these sugars upon the activity of streptomycin. The sugars were incorporated in nutrient agar, which was inoculated with a spore suspension of *B. subtilis*, and plates were poured. Metal cups were placed upon the solidified agar plates and the streptomycin solution was added. The results presented in Table IV show that those sugars, namely dextrose, levulose, and sucrose which yield acid diminish most markedly the action of streptomycin. Mannose, galactose and lactose, from which little or no acid is produced, have little effect upon the activity of streptomycin.

When *E. coli* was used as the test organism (Table V) it was found that acid was produced by this particular strain from all the sugars, but in the smallest amount from lactose. Likewise all the sugars but lactose gave a diminution in the effectiveness of streptomycin against this organism.

That the inactivating effect of glucose upon streptomycin is due to the production of acid has been further confirmed by the following experiment: when the pH of the test medium was held at 8.0 by the addition of a glycine buffer, the addition of glucose did not result in the inactivation of streptomycin. Glycine alone had no effect upon the activity of streptomycin.

In view of observed effects of cysteine, the

TABLE V
Effect of Sugars Upon Susceptibility of *E. coli*
to streptomycin

Sugar added*	pH after growth	Cup method zone size in mm
None	6.5	17.5
Dextrose	5.1	15.0
Levulose	5.1	16.0
Sucrose	5.0	15.5
Mannose	5.0	15.0
Galactose	5.2	15.2
Arabinose	5.2	17.0
Maltose	5.3	16.8
Lactose	6.1	18.0
Mannitol	5.2	16.5

* 1% in streptomycin agar

TABLE VI
Effect of Cevitamic Acid Upon Action of Streptomycin

Cevitamic acid	Activity, in dilution units per gram, against			
	<i>E. coli</i>	<i>A. aerogenes</i>	<i>S. aureus</i>	<i>B. subtilis</i>
0	100	100	50	800
0.4 mg/ml	10	10	5	100

TABLE VII
Effect of Sulphydryl Compounds on Action of Streptomycin

Sulphydryl compound	Zone size, mm	
	<i>B. subtilis</i> *	<i>E. coli</i> †
None	18.0	27.0
Cysteine (0.02 M)	10.0	8.0
Thioglycolate (0.04 M)	17.0	25.5
Monothioglycol (0.04 M)	—	22.0
Glutathione	18.0	—

* Streptomycin concentration 25 units/ml medium, nutrient agar

† Medium, streptomycin agar

effect of another reducing agent—cevitamic acid—upon the activity of streptomycin was studied. The tests were made by the agar-streak method, using nutrient agar at pH 6.8 and nutrient agar containing 0.4 mg of cevitamic acid per ml and adjusted to pH 6.8. The results (Table VI) show that cevitamic acid greatly diminishes the effectiveness of streptomycin. Tests for the production of acid from cevitamic acid of this concentration showed that no detectable acid was produced by the test organisms. The cause of inhibition by the cevitamic acid is thus not immediately apparent.

Effect of Sulphydryl Compounds According to Denkewalter, Cook and Tishler³ cysteine and β -mercaptoethylamine inhibited the action of streptomycin on *B. subtilis*, whereas thioglycolic acid was ineffective. A detailed study was made of this phenomenon, which fully confirmed their results and permitted the extension of their conclusions to include also the gram-negative *E. coli* (Table VII). It was also found that cysteine does not stimulate the production of acid by these organisms.

Thioglycolate agar (Brewer's) has been proposed as a medium for use in testing streptomycin for sterility. This medium contains not only thioglycolate, but also glucose,

and is far richer in nitrogenous constituents than the usual nutrient agar. Accordingly, the effects of these constituents upon the activity of streptomycin were studied. The results of several experiments (Table VIII) show that the presence of 0.2% of sodium thioglycolate in nutrient agar did not inactivate streptomycin. Whereas the presence of 1.0% of glucose diminished the bacteriostatic effect of streptomycin. Glucose plus thioglycolate had no greater effect than glucose alone. With an increase in the concentration of the nitrogenous constituents in the medium, streptomycin was found generally less effective, but the absence of inactivation by thioglycolate, diminution of activity by glucose, and absence of synergistic action of the 2 substances were again observed.

Effect of Ketone Reagents Brink, Kuehl and Folkers³ have reported that "when streptomycin was treated with a variety of carbonyl group reagents, complete inactivation was observed." They mentioned particularly hydroxylamine and semicarbazide, and have isolated the oxime and semicarbazone. To confirm their results, solutions of streptomycin were mixed with the ketone reagents and the mixture was tested by the cup method (Table IX). Certain amino compounds which are not ketone reagents, such as ethylenediamine and methylamine, were without effect upon the activity of streptomycin.

Age of Culture Streptomycin appears to have a more marked effect upon young actively growing cultures of bacteria than upon older cultures where the rate of growth has diminished. To confirm this, streptomycin was added to cultures of *E. coli* in nutrient broth which contained streptomycin, and after 8 hours' further incubation at 28°C, the number of viable cells were counted.

It was found that when streptomycin was

TABLE VIII
Effect of Thioglycolate and Glucose Upon Action of Streptomycin

Substance added	Dilution units against		
	<i>E. coli</i>	<i>S. aureus</i>	<i>B. subtilis</i>
None	100	50	1000
Glucose*	30	20	200
Thioglycolate	100	50	1000
Glucose+ thioglycolate	30	20	30

Nutrient agar—cup method

	Zone of inhibition, mm	
	<i>B. subtilis</i>	<i>E. coli</i>
None	16.5	16.7
Glucose	10.0	14.5
Thioglycolate	15.3	16.2
Glucose+ thioglycolate	10.0	15.7

Enriched agar—agar streak method

	Dilution units against		
	<i>E. coli</i>	<i>S. aureus</i>	<i>B. subtilis</i>
None	10	50	300
Glucose	5	10	10
Thioglycolate	20	20	200
Glucose+ thioglycolate	10	10	50

* Glucose added in 1% concentration, and thioglycolate in 0.2%

† Composition of enriched agar

Beef extract	5 g
Peptone	20 g
NaCl	5 g
Agar	20 g
Dist. water	1000 ml

added to cultures from 0 to 3 hours old, a 75% reduction in growth resulted when the concentration of streptomycin was 2 units per ml and a 95% reduction in growth when the concentration was 5 units per ml. In

cultures 24 to 48 hours old, the effect of streptomycin was less marked. A concentration of 2 units per ml caused a reduction in growth of 19%, and a concentration of 5 units per ml a reduction of 42%.

Discussion The antibacterial activity of streptomycin can be largely or completely neutralized or antagonized by various chemical agents. These include glucose and certain other sugars, an anaerobic environment, certain sulfhydryl compounds, and ketone reagents. In some cases, as in the action of sugars or the anaerobic environment, the effect on streptomycin can be traced to the acidity produced under these particular conditions. However, in the effect of cysteine, cevitamic acid, and of ketone reagents the

TABLE IX
Effect of Ketone Reagents Upon Action of Streptomycin (25 units/ml)

Ketone reagent	Zone—size, ml	
	<i>B. subtilis</i>	<i>E. coli</i>
None	18	25
Hydroxylamine (0.02 M)	10.5	8.0
Hydrazine (0.02 M)	—	10.0
Semicarbazide (0.02 M)	9.0	—
Phenylhydrazine (0.02 M)	13.0	8.0
Methylphenylhydrazine (0.01 M)	15.0	10.0

TABLE VI
Effect of Cevitamic Acid Upon Action of Streptomycin

Cevitamic acid	Activity, in dilution units per gram, against			
	<i>E. coli</i>	<i>A. aerogenes</i>	<i>S. aureus</i>	<i>B. subtilis</i>
0	100	100	50	800
0.4 mg/ml	10	10	5	100

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Effect of Sulphydryl Compounds According to Denkewalter, Cook and Tishler³ cysteine and β -mercaptoethylamine inhibited the action of streptomycin on *B. subtilis*, whereas thioglycolic acid was ineffective. A detailed study was made of this phenomenon, which fully confirmed their results and permitted the extension of their conclusions to include also the gram-negative *E. coli* (Table VII). It was also found that cysteine does not stimulate the production of acid by these organisms.

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It was found that when streptomycin was

TABLE I
Blood Platelets and Leukocytes in Heterophil Anaphylaxis of the Guinea Pig

Guinea pig No	Weight, g	Antisheep rabbit serum, cc/100 g body wt	Grade of shock	Blood platelets, vol in 100 cc of blood		Leukocytes, thous inds per cu mm of blood	
				Before inj	After inj	Before	After
1	500	0.10	Death in 2 min	1.20	0	—	—
2	510	0.02	No shock	1.30	0	12.4	10.8
3	570	0.07	Death in 1 min	1.20	0.05	14.8	5.0
4	535	0.01	No shock	0.85	0.85	12.3	11.8
5	530	0.02	" "	0.85	0.20	14.4	5.0
6	500	0.02	Slight dyspnea	0.70	0	18.9	7.8
7	490	0.10	Death in 1 min	0.80	0	—	—
8	510	0.015	No shock	0.60	0.05	12.0	11.2
9	590	0.10	Death in 1 min	0	0	—	—
10	940	0.05	No shock	0.70	0	—	—
11*	1,030	0.10	Death in 1 min	0.40	0	—	—
12*	1,000	0.15	Death in 2 min	0	0	—	—
13	900	0.10	Death in 1 min	—	—	—	—

* The last 3 guinea pigs were injected with 1 g of glycogen in the vein of the penis before the injection of antishock rabbit serum and the platelets were determined after the injection of glycogen and after the injection of serum.

able amounts of rabbit serum containing anti-sheep hemolysin. These serums were prepared by injecting erythrocytes of sheep into the rabbits, standard technics being followed. The hemolytic titer of the serum used in these experiments was 20,000 (that is, 0.00005 ml hemolyzed 0.5 ml of 2% sheep cells in the presence of 0.05 ml of pooled guinea pig serum as complement). Following intracutaneous injections of 0.10 ml of this serum into guinea pigs and dogs a notable hemorrhagic lesion appeared locally.

Five of the 13 guinea pigs used in these experiments were injected in the vein with 1 g of hepatic glycogen, dissolved in 5 ml of water, 3 minutes before the injection of the immune rabbit serum.

Four guinea pigs were injected with normal rabbit serum as controls and the volume of the platelets was determined before, and from 3 to 10 minutes after, the injection.

Different amounts of the immune rabbit serum were injected into 4 dogs anesthetized with pentobarbital sodium and with the femoral artery cannulated in order to determine the blood pressure. The volume of

platelets was determined before and after the injection.

All specimens of blood were drawn directly from the heart of the guinea pigs and from the jugular vein of the dogs. In all the guinea pigs that manifested shock the blood samples were taken immediately following the appearance of symptoms. In animals without shock the samples were taken from 3 to 10 minutes after the injection of serum.

For the determinations of the platelets the Van Allen method¹⁰ was used, in guinea pigs 2 ml of blood were taken in 3 ml of 3.8% solution of sodium citrate with 0.1% of formalin.

In several animals the modification of the leukocytes was studied and standard microscopic methods were used.

Results From the data presented in Table I it may be observed that the immune rabbit serum employed in these experiments is highly toxic for the guinea pig, since 0.07 ml for each 100 g of body weight injected into the

¹⁰ Van Allen, C. M., *J. Lab. and Clin. Med.*, 1926, 12: 292.

inhibition of streptomycin activity may be associated with the blocking of an active grouping in the molecule of the streptomycin. To explain the inactivation of the antibacterial properties of streptomycin by the blocking of a single group in its molecule cannot

be attempted at present due to its rather complicated structure. Until the chemistry of streptomycin is more clearly elucidated, it is difficult to present a suitable theory that would account for the various effects of streptomycin inactivation.

15269

Blood Platelets in Heterophil Anaphylaxis

A. GRANA * (Introduced by Hiram E. Essex)

From the Mayo Foundation, Rochester, Minn

The serum of rabbits into which erythrocytes of sheep have been injected produces toxic symptoms and death when injected into guinea pigs. The reason for this was explained when Forssman¹ and others^{2,3} announced the discovery and distribution of the so-called heterophil antigen. At this time it was found that the same antigenic substance which is present in erythrocytes of sheep is also present in the tissues of the guinea pig, dog and other species. Hence, the antibody produced in the rabbit in response to erythrocytes of sheep would also react with the antigen present in the tissues of the animal injected.

As the reactions exhibited by the animals following such injections of serum are almost identical with those of anaphylaxis, the phenomenon was designated heterophil anaphylaxis.

Later Taniguchi^{4,5,6} observed that the lungs of guinea pigs killed by such injections of serum were markedly hemorrhagic and both

lungs and trachea were filled with frothy fluid but were not markedly emphysematous as in true anaphylaxis. Furthermore Redfern⁷ observed that no contraction is produced in the perfused uterine muscle following the addition of anti-Forssman serum. I have confirmed these facts shown by previous investigators. Moreover I found in collaboration with Recarte⁸ that no increase of blood histamine was present in the acute shock induced in guinea pigs by the injection of anti-Forssman serum and no histamine was liberated when lungs of guinea pigs were perfused with such serums.

The experiments reported in the present paper were made with the aim of studying the modifications of the blood platelets in this particular type of shock. For this the volume of blood platelets was determined in guinea pigs and dogs before and after the injection of rabbit serum with high titer of antish sheep hemolysin. At the same time the influence of injections of hepatic glycogen on the course of this type of shock was observed, since this substance in certain doses produces a temporary disappearance of the platelets of the blood.⁹

Methods. Thirteen unanesthetized male guinea pigs weighing from 500 to 1,030 grams were injected in the vein of the penis with vari-

* Guggenheim Fellow, from the Institute of Experimental Medicine, Montevideo, Uruguay.

¹ Forssman, J., and Hirtz, Assen, *Biochem Z*, 1912, **44**, 336.

² Doerr, R., and Pick, R., *Biochem Z*, 1913, **50**, 129.

³ Sachs, H., and Nathan, E., *Z f Immunitätsforschung u exp Therap*, 1913, **19**, 235.

⁴ Taniguchi, Tenji, *J Path and Bact*, 1920, **23**, 364.

⁵ Taniguchi, Tenji, *J Path and Bact*, 1921, **24**, 217.

⁶ Taniguchi, Tenji, *J Path and Bact*, 1922, **25**, 77.

⁷ Redfern, W. W., *Am J Hyg*, 1926, **6**, 276.

⁸ Grana, A., and Recarte, P., *Rev Soc argent de biol*, 1945, **21**, 202.

⁹ Rocha e Silva, M., Grana, A., and Porto, A., *Proc Soc Exp Biol and Med*, 1945, **59**, 57.

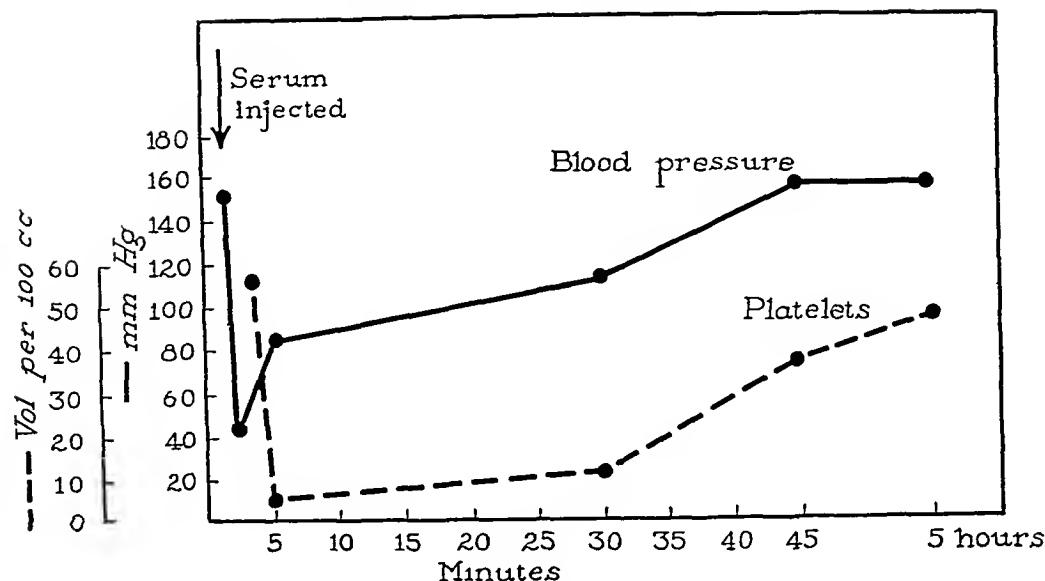


Fig 1

Platelets and blood pressure of a dog followed for 5 hours. As seen, 5 hours after the injection of 0.10 ml of immune rabbit serum for each kilogram of body weight the volume of platelets reached approximately the normal values.

values (Fig 1). In addition to the reduction of platelets there was a drastic reduction of the number of leukocytes in the dog (Table III).

Summary Serum of rabbits immune to sheep erythrocytes injected intravenously in appropriate doses in guinea pigs and dogs produced a drastic reduction of the concentration of blood platelets even though these quantities of serum provoked only moderate shock or no shock.

When the shock was deep, generally the platelets disappeared from the blood.

The injection of glycogen in doses that reduced the blood platelets in the guinea pig did not prevent the production of shock following the injection of the immune serum.

The experiments here presented show that there is not always a parallel relation between the decrease of blood platelets and the intensity of the pulmonary symptoms in the guinea pig or the decrease of arterial pressure of the dog in heterophil anaphylaxis.

15270

Subacute and Chronic Toxicities of Ascorbyl Palmitates

O. GARTH FITZHUGH AND ARTHUR A. NELSON

From the Division of Pharmacology, Food and Drug Administration, Federal Security Agency, Washington, D. C.

The fatty acid monoesters of l-ascorbic and d-isoascorbic acid prepared by Swern *et al.*¹ were suggested for use as antioxidants for fats and oils by Riemenschneider *et al.*² In

cooperation* with these investigators we have made a study of the subacute and chronic oral toxicities of the palmitates of these acids.

¹ Swern, D., Sturton, A. J., Turer, J., and Wells, P. A., *Oil and Soap*, 1943, 20, 224.

² Riemenschneider, R. W., Turer, J., Wells, P. A., and Ault, W. C., *Oil and Soap*, 1944, 21, 47.

* The Eastern Regional Research Laboratory of

TABLE II
Blood Platelets in Guinea Pigs Injected with Normal Rabbit Serum *

Guinea pig No	Weight, g	Normal rabbit serum, cc/100 g body wt	Blood platelets, volume in 100 cc of blood	
			Before	After
1	1,030	0 10	1 20	1 0
2	1,000	0 05	1 20	1 0
3	1,170	0 10	1 20	1 20
4	960	0 02	1 40	1 40

* None of these guinea pigs exhibited shock

TABLE III
Blood Platelets and Leukocytes in Heterophil Anaphylaxis of the Dog

Dog No	Weight, kg	Antisheep rabbit serum, cc/kg body wt	Grade of shock	Blood platelets volume in 100 cc of blood		Leukocytes, thousands per cu mm of blood	
				Before inj	After inj	Before	After
1	10	0 5	Death in 1 min	0 60	0	—	—
2	10 3	0 20	Severe shock, death after 90 min	0 90	0 (4 min) 0 2 (10 ") 0 5 (90 ")	17 0	16 26 28
3	18	0 10	Slight shock, blood pressure decreased 15 mm Hg	0 60	0 17	7 2	0 6
4	6 8	0 10	Moderate shock, blood pressure decreased 90 mm Hg, recovery in 90 min	0 55	0 05 (2 ") 0 12 (30 ") 0 35 (45 ") 0 45 (5 hr)	3 0	0 4 1 0 1 4 4 2

vein caused the death of the animal in one minute

The lungs of guinea pigs and dogs that died in the course of these experiments showed deep macroscopic hemorrhagic lesions. The microscopic examination showed bronchial exudate in the terminal bronchi and striking adventitial hemorrhage around the larger vessels.

The volume of platelets was always drastically reduced after the injection of 0.02 ml or more per 100 g of body weight of this immune rabbit serum in spite of the fact that injections of small quantities of serum did not produce shock (Table I).

On the other hand, as shown in Table I, 4 of the last 5 guinea pigs which were injected intravenously with 1 g of glycogen, which eliminated all or nearly all of the blood platelets, were killed by an injection, 3 minutes later, of immune rabbit serum.

The experiments described show that in this special type of anaphylaxis the decrease of the blood platelets does not parallel the degree of shock as Kopeloff and Kopeloff¹¹

found in true anaphylaxis.

The reduction of blood platelets under these circumstances raises the question whether the phenomenon observed is specific or nonspecific. As may be seen from Table II, there was no significant change in volume of the blood platelets when normal rabbit serum was injected into guinea pigs.

The sharp fall of the blood platelets after the injection of the immune rabbit serum perhaps depends on a reaction produced in the blood between the antisheep hemolysin injected and the Forssman antigen present in the platelets of the guinea pig.

As is shown in Table III, the same drastic reduction of platelets was observed in dogs after the injection of antisheep hemolysin, although the shock was slight or moderate in dogs 3 and 4. Five hours after the injection of 0.10 ml of immune rabbit serum for each kilogram of body weight the volume of platelets reached approximately the normal

¹¹ Kopeloff, Nicholas, and Kopeloff, Lenore M., *J. Immunol.*, 1941, 40, 471.

ences from the control animals of other experiments and their mortality rate was what would be expected for control animals in our colony

At 36 weeks, with the exception of the 1% d-isoascorbic acid group which showed no mortality, all groups of rats on the palmitate diets had fewer survivors than the group on the control diet. The figures for the percentage mortality were as follows: 5% d-isoascorbyl palmitate, 50%; 5% l-ascorbyl palmitate, 30%; 2% d-isoascorbyl palmitate and l-ascorbyl palmitate, 20% each, and the control 10%. The only group differing appreciably in mortality from the control, although not significantly with the small number of rats, was the 5% d-isoascorbyl group. Here there was a preponderance of deaths from acute pneumonia, but whether this should be attributed to the palmitates is doubtful.

All of the 60 rats started in the subacute series were autopsied, and 37 were microscopically sectioned. Lung, heart, liver, spleen, pancreas, stomach, small intestine, kidney, adrenal and testis were sectioned in all rats, while colon, lymph node, bone, bone marrow, thyroid and parathyroid were sectioned occasionally.

Two rats showed distinct lesions as a result of the palmitate feeding, and these were unusual. Both rats had been fed 5% l-ascorbyl palmitate for 36 weeks. Their urinary bladders were packed with numerous white mulberry stones up to 5 mm in diameter, one of which on crystallographic examination was found to consist of rods and needles of calcium oxalate monohydrate. Since oxalic acid has been reported⁴ to be an oxidation product of ascorbic acid this would seem to be a reasonable source of the oxalate in the stones. In the feeding of over 50 substances to several thousand rats in 2-year chronic toxicity studies we have not observed spontaneous bladder stone formation. Stones have been produced with relative frequency by the feeding of glycols⁵

and in one instance each by the feeding of barium chloride and saccharic acid. The last mentioned stone was composed of calcium oxalate, as were those caused by feeding glycols, the remaining stone was composed of barium sulphate in an organic matrix. In each rat with bladder stones one kidney and its ureter were markedly distended by the obstruction from the stones. Whitish material similar to the stones was seen in both kidney pelves of one rat. A small amount of colorless anisotropic crystalline calcareous material was seen in the parenchyma of one of the kidneys near the pelvis, this was the only kidney where such parenchymal crystalline material was seen. The kidneys in the 2 rats with urinary tract stones showed no inflammatory changes, although the bladders did show epithelial hyperplasia. Among the remaining 58 rats the surprising features were the almost total absence of calcareous material and of inflammatory changes within the kidneys and the lack of transitional examples in the gross appearance of the urinary tracts. Nor did the microscopic sections show any inflammatory changes or evidence of stone formation, with one exception, in a rat on 5% l-ascorbyl palmitate. There was a moderate degree of a focal, rather acute inflammatory process involving the interstitium and tubules of both cortex and medulla. Although this particular type of reaction has rarely been observed previously in our rats, it is not necessarily attributed to the palmitate, as unusual lesions do appear spontaneously in our rats from time to time.

Grossly, the viscera of the rats on the 5% palmitate diets, especially the l-ascorbyl palmitate, were slightly smaller than those of the controls and of the rats on 2% palmitate. Microscopically, except for the renal changes accompanying the 2 examples of stone formation and for the few spontaneous lesions that were observed, there was no outstanding difference among the 4 experimental groups and the control group. The amount of hemosiderin in the spleens of the group fed 5% palmitate was about half that in the remaining groups. The adrenals in all the experimental groups were slightly larger than those in the controls, however,

⁴ Borsook, H., Davenport, H. W., Jeffreys, C. E. P., and Warner, R. C., *J. Biol. Chem.*, 1937, **117**, 237.

⁵ Morris, H. J., Nelson, A. A., and Calvery, H. O., *J. Pharm. and Exp. Therap.*, 1942, **74**, 266.

TABLE I
Mean Gain in Weight of Male Rats Fed Diets
Containing Palmitates for Six Months

Substance	Dosage %	No of Animals	Mean Gain Wt g	Standard Error of Mean g
l-ascorbyl palmitate	2	8	350.6	± 17.1
	5	8	231.9	$\pm 22.3^*$
d-isoascorbyl palmitate	2	8	375.5	± 14.3
	5	7	269.6	$\pm 15.1^*$
d-isoascorbic acid	1	10	405.3	± 11.3
Control		9	394.7	± 12.6

* $p = < 0.01$

The results are reported in the following

Experimental For the subacute toxicity experiment 10 weanling male rats, selected at random with respect to litter mates from our colony of Osborne-Mendel strain, were placed on each of 6 diets. Ground commercial rat biscuits with 1% added cod liver oil served as the basic diet. The ascorbyl palmitates were mixed with the basic diet by means of a rotary batch mixer. Two groups of rats were placed on diets containing 2% and 5% concentrations of l-ascorbyl palmitate, two groups were placed on diets containing 2% and 5% d-isoascorbyl palmitate, one group was placed on a diet containing 1% d-isoascorbic acid and the sixth group received the control diet. Three rats were chosen from each of 6 litters and assigned to different diets as required for balanced incomplete blocks¹ of 3 with 6 treatments, where the blocks correspond to litters and treatments to diets. The design as a whole was replicated twice and thus a total of 60 rats was used. All animals were kept in individual cages in a room with controlled temperature and humidity and were given free access to their respective diets and water. Body weights and food consumption were determined at weekly intervals.

The U. S. Department of Agriculture furnished the palmitate monoesters and lard samples and partially defrayed the expenses of this study.

¹ Fisher, R. A., and Yates, F., *Statistical tables for biological, agricultural, and medical research*, Oliver and Boyd, Edinburgh, 1938.

In the chronic toxicity study 6 groups of 10 rats each were selected and assigned to their respective diets similarly to those in the subacute experiment. In addition to a 5% lard sample, with or without added palmitate, the ingredients of the diets were casein 18%, dextrose 61%, brewer's yeast 5%, salt mixture (U. S. P. XII No. 2) 4%, corn oil 3%, whole liver powder 2%, and cod liver oil 2%. The ration for each of the 6 groups contained 5% of one of the following lard samples,[†] namely, heat-treated lard containing 1% or 5% l-ascorbyl palmitate, heat-treated lard containing 1% or 5% d-isoascorbyl palmitate, heat-treated lard without added palmitate, and fresh lard. All diets were refrigerated at $-6 \pm 7^\circ \text{C}$.

Results, Subacute Toxicity This experiment was terminated at 36 weeks when all surviving animals were sacrificed. The data on the gains in weight during the fast growing period of the first 26 weeks (Table I) show that the animals on the 5% concentrations of the two palmitates grew significantly ($p = < 0.01$) less than those on the control diet. At the 2% levels the differences between experimental and control animals were not statistically significant ($p = 0.05$, or less, is significant for this experiment). Although the gains in weight of the animals on the 1% d-isoascorbic acid were slightly greater than those of the controls, these differences were not significant. During the next 10 weeks, however, this group grew significantly faster than the controls. Because of this fact and also because all animals of the group were living, they were continued over a 2-year experiment, at the end of 2 years the surviving animals on the d-isoascorbic acid had no gross or microscopic pathological differ-

[†] The palmitates were first incorporated in lard. The heat-treated lard samples with palmitates were prepared by heating fresh lard containing the palmitates at 100°C in a beaker with mechanical stirring and with free access to air for about 12 hours. The heat-treated lard without added palmitate was prepared by heating fresh lard at 100°C with washed air bubbling through the lard for 8 hours. Peroxide values showed that the heat-treated lard without the added palmitate was rancid.

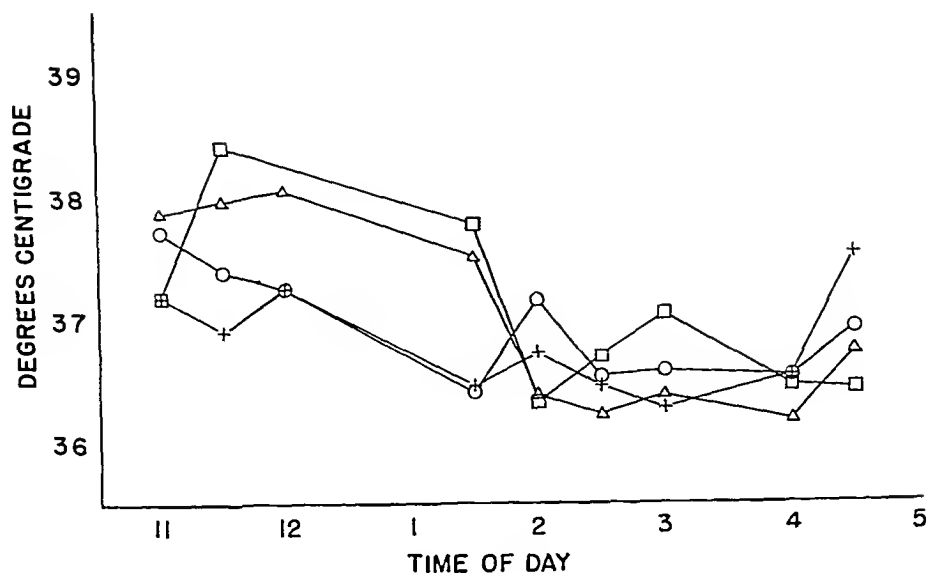


Fig 1

Body temperatures of 4 normal rats taken at intervals during the day

temperatures of rats which are free in their cages and are neither excited nor subjected to a reduced rate of heat loss

The thermocouples are made from 20 inch lengths of enamel and cotton covered iron and constantan wires, B & S gauge No 30, which are cleaned and fused together at one end. The junction is then covered with a bead of hard solder about 2 mm in diameter which is smoothed with a file, and a $1\frac{1}{2}$ inch length of rubber catheter tubing, size No 8 French, is passed over the wires and pulled down tightly against the solder bead. A 10 mm length of rubber tubing, $7/32$ inch outside diameter, $1/8$ inch wall, fitted over the other end of the catheter tubing, serves to anchor the end of a 15 inch length of light steel helical pull spring, $1/4$ inch in diameter, through which the lead wires pass to the outside of the cage. The purpose of this spring is to protect these wires from attack by the rat.

It is important that proper attention be paid to the gauge of the thermocouple wires and to the size of the catheter tubing. If the assembled junction is too limber it will be forced out of the rectum during defecation, and if it is too stiff it will cause ulceration and perforation of the colon. The

stiffness should be regulated by proper choice of the catheter tubing, and not by the gauge of the wires, which should be quite fine.

The thermocouples are attached outside the cage with silver solder to heavier wires of iron and of constantan which lead to a selector switch* making it possible to put a number of rats on test at the same time. The connections with the switch should be enclosed in a box to protect them from air currents.

The wire leading from the constantan side of the selector switch is attached to the constantan side of another thermocouple which is immersed in a reference bath of oil. This bath is mechanically stirred and electrically heated to a temperature of about 38°C , and it contains a calibrated thermometer. The iron sides of the reference junction and of the selector switch are attached through a double pole switch to the terminals of a sensitive galvanometer (550 μv per mm, internal resistance 21 ohms) and a precision wound variable resistance with a total value of 70 ohms is introduced into the lead from

* Selector switches designed for use with thermocouples are available commercially and are preferable to the ones generally used in electronic equipment.

there was no microscopic difference. The testes were undamaged, with rare exceptions.

Results, Chronic Toxicity When the gains in weight were analyzed for the experimental and control animals for the first 24 weeks and for the first year, the differences were not statistically significant for any group. During the fast growing period individual growth curves of litter mates were enough alike to be considered identical. Growth curves of the experimental groups showed no significant deviation from the control, or from each other.

Each of the 4 rats which died during the first year was from a different group, therefore, their deaths were not related to treatment. During the second year deaths were distributed over the whole period and could not be attributed directly to the effect of the palmitates. At the termination of the experiment no group had a death rate greater than that of the control.

Histopathological study similar to that of the subacute toxicity series was made of the tissues of 36 of the 60 animals and showed no outstanding gross or microscopic difference between any of the experimental groups and the control group, nor were there any distinct lesions attributable to or prevented by the palmitates. The one group of rats which did differ slightly from all the others was that fed heat-treated lard without the added palmitates. In this group, the livers usually had a slight brown or chocolate tint rarely

seen in the other groups. Microscopically, lymphocytic infiltration more frequently accompanied the small amount of focal renal tubular atrophy seen equally in all the groups, testicular atrophy was greater in degree than in all other groups, and lung involvement by chronic infectious processes and by lymphosarcoma was less frequent than in the rats whose diet included lard with added palmitates or unheated lard. However, an analysis of these data from the small number of animals in this experiment does not show a statistically significant difference.

Hematological studies made at intervals during the first year of the experiment showed no toxic effect on the blood from any of the chronic dosages of the palmitates.

Summary When l-ascorbyl palmitate and d-isoascorbyl palmitate were fed subcutely for 9 months in concentrations of 2% and 5% of the diet, only the 5% levels were toxic. The growth rates of rats on the 5% levels were significantly retarded and bladder stones were produced in 2 animals on the 5% l-ascorbyl palmitate. d-Isoascorbic acid in a concentration of 1% of the diet had no deleterious effect. Chronic toxicity studies for 2 years indicate that l-ascorbyl and d-isoascorbyl palmitate are not toxic in concentrations of 0.25% (2500 ppm), or less, of the total diet of rats. Heat-treating fresh lard containing the palmitates before adding it to the diet had no effect on toxicity.

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Use of Thermocouples for Determination of the Rectal Temperature of Rats

DAVID M. TENNENT AND MARTHA A. MELOY (Introduced by H. Molitor)

From the Merck Institute for Therapeutic Research, Rahway, N. J.

When measurements of the rectal temperatures of rats were made in this laboratory with thermometers or with thermocouples while the animals were held in the investigator's hand, the readings were unstable and frequently changed by as much as one degree

centigrade during the space of a few minutes. The results were also unsatisfactory when mechanical holders made of wire mesh were used, since the rats became unduly excited. To avoid these difficulties a method has been devised, using thermocouples, for taking the

the selector switch so that the sensitivity of the galvanometer can be adjusted

After calibration, this circuit will measure the differences between the temperatures of the rats and that of the reference bath to $\pm 0.05^\circ\text{C}$. Each junction must be individually calibrated and will serve during daily use for about 10 days, failure being caused by rusting of the iron wire near the tip.

Specially designed potentiometers for use with iron-constantan thermocouples and containing automatic reference junction compensators are available commercially. Instruments of this type will afford greater convenience of measurement, but less sensitivity, than the circuit which has been described.

For use, the junctions are lubricated and inserted rectally to a depth equal to the length of the catheter tubing, and the end of the steel spring is firmly fastened with adhesive tape to the base of the rat's tail, which is first covered with a layer of gauze to minimize irritation. The adhesive tape is then covered with a piece of 40 mesh brass

gauze (about $2\frac{3}{4} \times \frac{5}{8}$ in after the edges have been doubled over) which is held in place with 2 or 3 turns of wire.

After insertion of the thermocouples, the rats are returned to their cages, and the first readings may be taken about 30 minutes later. The junctions may be left in place for considerable periods of time. In the severest test to which the apparatus has been put, the temperatures of 9 rats were taken over 10 7-hour periods during 13 consecutive days. When the rats were sacrificed at the end of this time no lesions of the rectum or anus were found. Temperatures taken on a typical day are presented in Fig. 1.

Summary 1 An apparatus for the measurement of the rectal temperatures of rats while they are free in their cages has been described. 2 An observed instability of readings which was presumably caused by reduction of heat loss or by excitation is avoided with this technic. 3 Typical results obtained at intervals during the day have been presented.

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Blood Concentration of P-chloro-xyleneol in Man Following Parenteral, Percutaneous and Rectal Application *

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It has been shown^{1,2} that halogenized phenols and especially p-chloro-xyleneol (3,5-dimethyl, 4-chlorophenol, referred to subsequently as CX), and its derivatives exert a chemotherapeutic effect in man, in urogenital and general septic infections. Because of local irritation large amounts of these sub-

stances cannot be administered parenterally. We have, therefore, availed ourselves of experience gained in estrogenic hormone therapy³ and applied CX percutaneously. Concurrently, CX is introduced rectally. In this paper it will be shown that free CX appears in blood of patients treated with CX by various routes of administration. CX was determined in blood according to Zondek *et al.*⁴

* Aided by a grant from the Rockefeller Foundation.

† We are deeply obliged to Drs. Bromberg, Brzezinski, Weber and Sulman for assistance rendered.

1 Zondek, B., *Nature*, 1942, **149**, 334.

2 Zondek, B., *J. Urology*, 1942, **48**, 747.

3 Zondek, B., *Klin. Wschr.*, 1929, **48**, 2229, *Schw. Med. Wschr.*, 1935, **65**, 1168.

4 Zondek, B., Shapiro, B., and Hestrin, S., *Bioch. J.*, 1943, **37**, 589.

1 *CX in blood after intramuscular injection* A 10% solution of CX in olive oil containing 2% anesthesin was employed. Other vehicles proved unsatisfactory. Doses of 10-20 cc (1 and 2 g CX respectively) were injected intragluteally into 12 healthy and diseased subjects. The blood was analyzed 2 to 6 times during the 48 hours following administration of the drug. In 3 cases no demonstrable amount of CX was found in the blood following administration of 1 g of CX, but in the other cases 1 mg% of CX was found 2-4 hours after the injection. The highest value found was 4 mg% 12 hours after injection in a case of hepatitis. With 2 g CX the blood content of CX was higher. In one case, 12 mg% was found 1 hour after injection, in 2 other cases 15 mg% and 3 mg% respectively 2 hours after injection. After 24 and 36 hours a concentration of 1 mg% was still demonstrated, whereas after 48 hours the blood contained no CX.

2 *Gastro-intestinal or rectal absorption of CX* a *Stomach* CX on the tongue produces a numb sensation followed by burning. The oral administration of large quantities of this substance is therefore difficult. In one case it was possible to administer 10 g of CX dissolved in oil by this route without untoward after-effects. Blood analyses in this case gave the following results: after 1 hour—2 mg%, after 24 hours—1.4 mg%, after 48 hours—0. The blood concentration of CX after oral administration of 10 g does not exceed that obtained following intramuscular administration of 2 g of CX. We assume that this is due to removal of CX from the alimentary tract by the vena portae to the liver where by combination with glucuronate and sulfate it is rapidly inactivated.[†]

b *Rectum* CX in 10% oil solution with 2% anesthesin can be administered rectally without injurious effect. Doses of 20 cc were administered once and twice daily, with a narrow catheter, backflow being prevented by closing the catheter with a clamp. Three patients were treated in this way. Follow-

ing administration of 20, 25 and 40 g by this route, the following blood values were recorded: after 2 hours—traces, 1 mg% and 3 mg% respectively, after 24 hours—1 mg% and traces in the other cases, after 48 hours—traces in all 3 patients.

3 *Percutaneous administration* Preliminary experiments indicated that large amounts of CX (10-30 g per day) can be administered percutaneously to man with no toxic effect. Eleven patients were treated percutaneously with 40% CX in alcohol-oil solution prepared by dissolving 100 g of CX in 100 cc ethanol and then adding an equal amount of olive oil gradually under continuous agitation. The results show that following percutaneous administration of a dose of 2 g, CX did not appear in demonstrable amount in the blood. After 5 g, traces were found, and after a dose of 8 g, 1 mg% CX was found after 3 hours, and 4 mg% after 24 hours. Following administration of larger doses (20 g), 4 mg% CX was demonstrable in the blood within one-half hour of the application and 1 mg% was still demonstrable in free form even after 72 hours.[‡]

CX is thus resorbed by the skin, passes thence into the blood, and persists there for considerable periods of time. The prolonged presence of CX in the blood can probably be ascribed to the slow absorption of CX from the skin which thus acts as a depot.

4 *CX in the skin* In conclusion it is of interest to note that skin treated with CX solutions or ointments remains sterile for 12 hours, a finding which in view of the known high disinfectant potency of CX is not unexpected. In performance of gynecological operations (Z) the skin of the hypogastrium was vigorously anointed with 40% CX in oil-alcohol solution 2-16 hours before operation. In one case 500 cm² of the skin was anointed

[‡] Unfortunately, we were unable to carry out similar experiments with CX ointment, as our original American lanolin material was exhausted. In view of the greater bacteriostatic power of blood of patients who had received this CX lanolin as compared with patients treated with CX oil-alcohol solution it seems possible that the concentration of CX in the blood of these patients was correspondingly higher.

[†] Liver inactivates CX as will be reported elsewhere.

with 4.0 g CX 2 hours before operating. During operation 3 cm² of skin and subcutaneous tissue was removed and later analyzed. Without subcutaneous tissue it contained 2.5 mg CX, i.e. 10.4% of that applied to the area. In another case treated with 4.0 g CX on an area of 450 cm², 0.3 mg CX was found 15 hours after the incision in 2.0 cm² skin, i.e. 1.7% of the CX applied. In the subcutaneous adipose tissue only 1-2 mg per g tissue were found. The findings show that CX penetrates into and through the skin.

Summary The presence of free p-chloro-

xyleneol (CX) in the blood shows that this substance is resorbed after parenteral, oral, rectal, as well as percutaneous application. Only 1% of the amount of CX applied was detected in the blood. In percutaneous treatment, the skin acts as a depot from which CX is slowly resorbed into the blood, and as a result a prolonged circulation of CX in active form in the blood is obtained. The prolonged circulation of CX in the blood in active form following different routes of administration renders its chemotherapeutic efficacy in man comprehensible.

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15273

Influence of Thiourea on Organ Weights of Rats as Related to Food Intake

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Thyroid hypertrophy is invariably observed following the administration of antithyroid drugs but the influence on other body organs is not definitely established¹. Since it is known that thiourea or thiouracil will reduce food intake^{2,3} and since inanition alone will alter organ weights, the present investigation was undertaken to determine the influence of reduced food intake alone on organ weights. Therefore, organ weights of thiourea-fed,

pair-fed and ad-libitum-fed rats were compared.

Male rats of the Long-Evans strain, ranging from 139 to 162 days in age, were used. These animals were kept in metabolism cages for the measurement of daily food intake. Thiourea was fed as half of one percent of the stock diet for a period of 20 to 27 days. At autopsy the fresh weight of the pituitary, adrenals, thyroid, seminal vesicles (including the coagulating gland and any contained fluid), kidneys and liver was recorded. The anterior pituitary was assayed for gonadotropic hormone content in the same manner as reported previously.²

Average food consumption per rat during the first 20 days totaled 258 g for the thiourea-fed rats as compared with 340 g for normal rats eating ad libitum. Furthermore the thiourea-fed rats exhibited a body weight

¹ Riker, W. F. and Wescoe, W. C., *Am. J. Med. Sc.* 1945, **210**, 665.

² Williams, R. H., Winglass, A. R., Bissell, G. W. and Peters, J. B., *Endocrinology*, 1944, **34**, 317.

³ Leitham, T. H., *Endocrinology*, 1945, **36**, 98.

⁴ Astwood, E. B., Sullivan, T., Bissell, A. and Tislowitz, R., *Endocrinology*, 1943, **32**, 210.

⁵ Hughes, A. M., *Endocrinology*, 1944, **34**, 69.

⁶ Gordon, A. S., Goldsmith, E. D., Chrappier, H. A., *Lab. Proc.* 1945, **4**, 25.

TABLE I
Influence of Thiourea on Organ Weights of Rats

Treatment (No of rats)	Avg organ wt g		Avg organ wt g per 100 g body wt		
	Thiourea fed (20)	Normal pair fed (20)	Thiourea fed (20)	Normal pair fed (20)	Normal ad lib fed (20)
Pituitary	0 009	0 009	0 004	0 003	0 003
Adrenals	0 025	0 030	0 010	0 011	0 010
Thyroid	0 036	0 022	0 014	0 008	0 008
Testes	2 899	2 856	1 126	1 020	1 063
Seminal vesicles	0 694	1 082	0 267	0 382	0 377
Kidney	2 533	2 545	0 977	0 919	0 891
Liver	10 082	10 525	4 057	3 772	3 984

TABLE II
Gonadotrophic Potency of Rat Pituitaries

Donors		Recipients			
		No of rats	Avg body wt	Avg organ wt — mg	
Treatment	Amt A P			Ovaries	Uteri
	mg		g		
—	—	10	55.1	13.4 ± 1.0*	24.7 ± 1.4
Thiourea	8.9	13	59.4	91.0 ± 7.5	72.1 ± 7.2
None—pair fed	8.5	13	57.2	94.0 ± 6.5	68.7 ± 4.2

$$* \text{Mean deviation of mean} = e = \sqrt{\frac{\sum d^2}{n(n-1)}}$$

loss averaging 46 g whereas the normal rats gained 17 g. The pair-fed normal controls lost only 19 g in weight although food intake was restricted to that of thiourea-fed rats.

Examination of organ weights revealed the anticipated thyroid hypertrophy but the weights of the adrenals, testes, kidney and liver were not affected. The pituitaries were slightly heavier following thiourea feeding but the increase was not significant. Furthermore, the degree of food restriction imposed on the rats pair-fed with the thiourea-fed rats was not sufficient to alter the normal organ weight/body weight ratio (Table I). Other investigators have also indicated that the above organs do not change in weight in animals receiving antithyroid drugs.^{2,3,7-9} However, Baumann and Marine¹⁰ have re-

ported a definite decrease in adrenal weight due to cortical atrophy in the presence of slight medullary hypertrophy whereas Kennedy and Purves¹¹ observed adrenal hypertrophy. An increase in pituitary, kidney and liver size has also been reported to occur when thiourea or thiouracil is administered.⁸ Although our data do not reveal an influence of thiourea on most organs over the 20-day period we have consistently obtained a reduction in seminal vesicle weight (Table I). It was of interest to find that gonadotrophic hormone of the anterior hypophysis of thiourea-fed and pair-fed rats did not differ although the decrease in seminal vesicle weight suggested a decrease in release of luteinizing hormone (Table II).

Summary The reduction in food intake caused by the addition of a half of one percent of thiourea to the diet for 20 days did not influence organ weight/body weight ratios in spite of a body weight loss. In addition to thyroid hypertrophy, a decrease in

⁷ Richter, C. P., and Clisby, K. H., *Arch. Path.*, 1942, **33**, 46.

⁸ Leblond, C. P., and Hoff, H. E., *Endocrinology*, 1944, **35**, 229.

⁹ Goldsmith, E. D., Gordon, A. S., and Chappier, H. A., *Endocrinology*, 1945, **36**, 364.

¹⁰ Baumann, E. J., and Marine, D., *Endocrinology*, 1945, **36**, 400.

¹¹ Kennedy, J. H., and Purves, H. D., *Brit. J. Exp. Path.*, 1941, **22**, 241.

seminal vesicle weight was evident after feeding and pair-fed rats did not differ. Pituitary, ing thiourea, although the gonadotropic testis adrenal kidney and liver weights were hormone content of the pituitary of thiourea- not significantly changed.

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Temperature Coefficients of Hemolysis of a Few Types of Nucleated Erythrocytes *

F R HUNTER (Introduced by A A Hellbaum)

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Jacobs *et al*¹ have presented a series of hemolysis of various types of mammalian data giving temperature coefficients for the erythrocytes in non-electrolytes. These data

TABLE I
Temperature Coefficients for Hemolysis in Isosmotic Solutions

Type of blood	Penetrating substance	Time in seconds for 80% hemolysis					Mean Q_{10} 10° 50° (except when enclosed in parentheses)
		10°	20°	30°	40°	50°	
Turtle	H ₂ O	64	55	46	40	28	123
	Urea	99	88	87	72	53	117
	Ethylene glycol	107.0	41.4	22.2	11.6	6.7	200
	Methyl alcohol	74	80	64	52	38	(128)
	Ethyl "	85	70	53	50	39	122
	Propyl "	97	96	78	61	48	(126)
Chicken	H ₂ O	38.2	21.3	13.6	8.5	5.0	166
	Urea	879.0	476.0	322.0	134.0	71.4	187
	Ethylene glycol	85.8	42.2	23.6	12.2	7.2	186
	Glycerol		1456.0	455.0	150.0	81.5	(261)
	Methyl alcohol	34.8	20.8	13.1	8.0	4.6	166
	Ethyl "	35.7	20.5	14.1	7.4	4.4	169
Monk Fish	Propyl "	37.4	21.2	13.5	8.4	4.4	170
	H ₂ O	116.0	73.7	62.3	48.5	6.5	206
	Urea	1623.0	551.0	299.0	178.0	75.3	216
	Glycerol	2625.0	905.0	342.0	143.0	61.9	255
	Methyl alcohol	449.0	346.0	103.0	23.2	10.9	253
	Propyl "	171.0	107.0	79.2	11.3	5.2	239
Dog Fish	H ₂ O	50.0	24.8	15.4	8.4	5.1	177
	Urea	1030.0	518.0	264.0	128.0	65.7	199
	Ethylene glycol	117.0	55.3	25.8	13.8	8.3	194
	Glycerol	2515.0	1114.0	724.0	293.0	141.0	205
	Methyl alcohol	41.7	27.8	14.4	7.9	5.1	169
	Ethyl "	43.8	27.6	13.7	7.6	5.1	171
Barn Skite	Propyl "	50.9	29.3	15.3	7.0	4.2	187
	H ₂ O	65.4	32.8	19.7	10.7	4.7	193
	Urea	1132.0	309.0	163.0	78.2	16.8	287
	Ethylene glycol	169.0	47.6	33.6	14.0	7.6	217
	Glycerol	2593.0	599.0	287.0	138.0	44.3	277
	Methyl alcohol	45.2	19.8	14.0	8.5	4.0	183
Barn door Skite	Ethyl "	57.5	25.0	14.2	9.1	3.1	208
	Propyl "	57.7	28.9	9.9	4.9	1.8	238
	H ₂ O	113.0	46.6	24.7	14.3	6.7	203
	Urea	753.0	393.0	142.0	69.4	27.5	229
	Ethylene glycol	183.0	75.7	42.7	27.1	11.2	201
	Glycerol	2756.0	883.0	315.0	194.0	80.2	242
	Methyl alcohol	81.9	50.1	25.3	13.9	5.6	196
	Ethyl "	86.6	42.5	22.1	12.2	5.1	203
	Propyl "	103.0	56.8	25.0	9.7	3.8	228

Avg values based on 1421 individual observations

* The experiments herein reported were performed in the Zoology Department Rhode Island State College Kingston, R I
by Jacobs M H Glassman, H N and Purport,

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Pituitary	0.009	0.009	0.004	0.003	0.003
Adrenals	0.025	0.030	0.010	0.011	0.010
Thyroid	0.036	0.022	0.014	0.008	0.008
Testes	2.899	2.856	1.126	1.020	1.063
Seminal vesicles	0.694	1.082	0.267	0.382	0.377
Kidney	2.533	2.545	0.977	0.919	0.891
Liver	10.082	10.525	4.057	3.772	3.984

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loss averaging 46 g whereas the normal rats gained 17 g. The pair-fed normal controls lost only 19 g in weight although food intake was restricted to that of thiourea-fed rats.

Examination of organ weights revealed the anticipated thyroid hypertrophy but the weights of the adrenals, testes, kidney and liver were not affected. The pituitaries were slightly heavier following thiourea feeding but the increase was not significant. Furthermore, the degree of food restriction imposed on the rats pair-fed with the thiourea-fed rats was not sufficient to alter the normal organ weight/body weight ratio (Table I). Other investigators have also indicated that the above organs do not change in weight in animals receiving antithyroid drugs.^{2,3,7-9} However, Baumann and Marine¹⁰ have re-

ported a definite decrease in adrenal weight due to cortical atrophy in the presence of slight medullary hypertrophy whereas Kennedy and Purves¹¹ observed adrenal hypertrophy. An increase in pituitary, kidney and liver size has also been reported to occur when thiourea or thiouracil is administered.⁸ Although our data do not reveal an influence of thiourea on most organs over the 20-day period we have consistently obtained a reduction in seminal vesicle weight (Table I). It was of interest to find that gonadotropic hormone of the anterior hypophysis of thiourea-fed and pair-fed rats did not differ although the decrease in seminal vesicle weight suggested a decrease in release of luteinizing hormone (Table II).

Summary The reduction in food intake caused by the addition of a half of one percent of thiourea to the diet for 20 days did not influence organ weight/body weight ratios in spite of a body weight loss. In addition to thyroid hypertrophy, a decrease in

⁷ Richter, C. P., and Chisbry, K. H., *Arch. Path.*, 1942, **33**, 46.

⁸ Leblond, C. P., and Hoff, H. E., *Endocrinology*, 1944, **35**, 229.

⁹ Goldsmith, E. D., Gordon, A. S., and Charney, H. A., *Endocrinology*, 1945, **36**, 364.

¹⁰ Baumann, D. J., and Marine, D., *Endocrinology*, 1945, **36**, 400.

¹¹ Kennedy, J. H., and Purves, H. D., *Brit. J. Exp. Path.*, 1941, **22**, 241.

of Bakersfield and Arvin. He arrived in Stockton (San Joaquin County) on September 4 or 5 1945, and lived at an auto trailer camp about 6 miles east of the city. On the morning of September 11 he was observed to be irrational and to walk with a stumbling gait, and was hospitalized with a diagnosis of acute encephalitis of undetermined etiology. Examination of the spinal fluid on admission showed the following: 10 red cells, 20 white cells, Pandé's reaction +, Gram stain for organisms negative, sugar 81.4 mg per 100 cc, chloride 720 mg per 100 cc, Kolmer's test 4 +, colloidal gold curve 0-0-1-2-1-0-0-0-0. Blood examination on admission showed hemoglobin 83 percent, white blood cells 20,900 polymorphonuclears, 76 lymphocytes, 14 mononuclears, 10 stab cells, 5, Kolmer and Kline tests both 4 +. The temperature at the time of admission was 107.4°, under treatment with medication and icecaps it dropped to 103° by the 3rd day, then rose to 105.3° at the time of death on the 4th hospital day. On post-mortem examination there was found some passive congestion of the lungs, a few scars in the liver, and an acute congestion and severe edema of the brain, the rest of the organs showed nothing unusual.

A 20 percent suspension of brain tissue in 10 percent normal rabbit serum broth was prepared and spun at 1500 rpm for 15 minutes in an angle centrifuge. The supernatant fluid was inoculated intracerebrally (0.1 ml) into 2 guinea pigs, intraperitoneally (0.03 ml) into 8 mice 14 days of age, and by the combined intracerebral (0.03 ml) and intraperitoneal (0.1 ml) routes into 8 mice 20 days of age. Neither guinea pig showed evidence of infection during the one month observation period. Of the 14-day-old mice, one was found dead on the 8th day and one on the 9th day, 2 were ill on the 9th day and were found dead on the 10th day, and 4 survived and were discarded on the 21st day. Of the 20-day-old mice, one was found dead on the 5th day and the remaining 7 were obviously ill on the 7th day, showing ruffled fur, hunching, tremors, and ataxia. Four of these animals were killed, and their brains were pooled

and passaged by combined intracerebral-intraperitoneal inoculation into 14- to 15-day-old mice; the remaining 3 animals were killed on the 12th day and their brains similarly inoculated in 15-day-old mice. In both cases the passage mice showed ruffling of the fur, convulsions, tremors, ataxia and paralysis of hind or fore limbs after an incubation period of 4 to 6 days.

Aerobic and anaerobic bacteriologic cultures of infected brains were either sterile or occasionally showed the presence of a few saprophytic bacteria. Since Berkefeld N and W and Seitz EK filtrates were bacteriologically sterile yet highly pathogenic for mice, the agent was regarded as a virus. For convenience, it has been designated the Winkler strain.

In an attempt to identify the agent, guinea pigs and hamsters were inoculated intracerebrally (0.1 ml), intraperitoneally (0.5 ml) or by both routes combined with a 1 percent suspension of brains from the 3rd mouse passage. Three animals were inoculated by each route, all survived without showing any evidence of infection during a 3-week observation period. The lack of pathogenicity for guinea pigs was considered possibly to rule out several viruses, and the failure to produce apparent infection in hamsters to rule out others, including the St. Louis virus, to which this species has been reported as susceptible^{5,6}. Nevertheless on the assumption that newly isolated strains of a virus might fail to produce obvious infection in species normally susceptible to highly passaged laboratory strains of the same virus, neutralization tests were done using the Winkler virus and rabbit or guinea pig immune sera to the Eastern equine encephalomyelitis, Western equine encephalomyelitis, lymphocytic choriomeningitis, Japanese B, St. Louis (Hubbard strain) and Winkler viruses, and to an unidentified virus isolated from mosquitoes of California by Hammon.

⁵ Brown, G. O., Muether, R. O., Mezera, R. A., and LeGier, M., *Proc. Soc. Exp. Biol. and Med.*, 1941, **46**, 601.

⁶ Lennette, E. H., *Proc. Soc. Exp. Biol. and Med.*, 1941, **47**, 178.

have recently been utilized in an attempt to relate species differences in permeability to possible chemical differences in the cell membrane (Dziemian² and Ballentine³)

Because of their possible use in future studies, and for comparative purposes, the data in Table I are presented giving temperature coefficients of hemolysis of nucleated erythrocytes of several species

The technic employed in these experiments was essentially that used by Jacobs *et al*⁴ In general, the temperature coefficients of hemolysis for these nucleated erythrocytes

are of the same order of magnitude as those obtained using mammalian red cells These data are too fragmentary to justify a detailed analysis However, certain comparisons can well be made with data described by Jacobs and Glassman⁴ These authors point out that in fishes (elasmobranchs and teleosts) the permeability to ethylene glycol is high, in birds the permeability to ethylene glycol and glycerol is very great and nearly equal, and much less to urea, except in the duck and chicken, and permeability to urea is great in reptiles The present data are in complete agreement Ethylene glycol permeability is high in the four fishes studied, urea and glycerol permeability is high in the chicken, and the turtle exhibits a very high permeability to urea

Arthur K, *J Cell and Comp Physiol*, 1935, **7**, 197

² Dziemian, Arthur J, *J Cell and Comp Physiol* 1939, **14**, 103

³ Ballentine Robert, *J Cell and Comp Physiol*, 1944 **23** 21

⁴ Jacobs, M H, and Glassman, H N, *Biol Bull*, 1937, **73**, 387

15275

Isolation of St Louis Encephalitis Virus from a Fatal Human Case in California*†

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Hammon and Reeves¹ have recently reported recovery of the St Louis encephalitis virus from naturally infected mosquitoes (*Aedes dorsalis* Meigen) caught in Kern County California, in 1944, the first isolation

of this virus from any source in this state

The present report describes the isolation of the St Louis virus from a fatal human case in California This is the first time the virus has been isolated from any vertebrate host outside the endemic St Louis area, and furnishes additional, more direct, evidence to that obtained from serologic studies,²⁻⁴ of the activity of this virus in California

The patient was an itinerant agricultural worker who, during the past year, had resided in Kern County in the neighborhood

* The work on which this paper is based was conducted with the support and under the auspices of the International Health Division of The Rockefeller Foundation in cooperation with the California State Department of Public Health

† The author is indebted to Dr John J Sippy, District Health Officer of the San Joaquin Local Health District, for clinical and epidemiologic data, and to Dr Elmer W Smith, Pathologist to the San Joaquin General Hospital, for information on the clinical and post mortem findings and for furnishing pathologic material for examination

¹ Hammon, W McD, and Reeves, W C, *Am J Pub Health*, 1945, **35**, 994

² Wainns, H L, and Hawley, C J, *Am J Pub Health*, 1939, **29**, 781

³ Howitt, B T, *Am J Pub Health*, 1939, **29**, 1083, *ibid*, 1942, **32**, 503

⁴ Hammon, W McD, Reeves, W C, and Galindo, P, *Am J Hygiene*, 1945, **42**, 299

of Bakersfield and Arvin. He arrived in Stockton (San Joaquin County) on September 4 or 5 1945, and lived at an auto trailer camp about 6 miles east of the city. On the morning of September 11 he was observed to be irrational and to walk with a stumbling gait, and was hospitalized with a diagnosis of acute encephalitis of undetermined etiology. Examination of the spinal fluid on admission showed the following: 10 red cells, 20 white cells, Pandv's reaction +, Gram stain for organisms negative, sugar 81.4 mg per 100 cc, chloride 720 mg per 100 cc, Kolmer's test 4 +, colloidal gold curve 0-0-1-2-1-0-0-0-0. Blood examination on admission showed hemoglobin 83 percent, white blood cells 20,900, polymorphonuclears 76, lymphocytes 14, mononuclears 10, stab cells 5, Kolmer and Kline tests both 4 +. The temperature at the time of admission was 107.4°, under treatment with medication and icecaps it dropped to 103° by the 3rd day, then rose to 105.3° at the time of death on the 4th hospital day. On post-mortem examination there was found some passive congestion of the lungs, a few scars in the liver, and an acute congestion and severe edema of the brain; the rest of the organs showed nothing unusual.

A 20 percent suspension of brain tissue in 10 percent normal rabbit serum broth was prepared and spun at 1500 rpm for 15 minutes in an angle centrifuge. The supernatant fluid was inoculated intracerebrally (0.1 ml) into 2 guinea pigs, intraperitoneally (0.03 ml) into 8 mice 14 days of age, and by the combined intracerebral (0.03 ml) and intraperitoneal (0.1 ml) routes into 8 mice 20 days of age. Neither guinea pig showed evidence of infection during the one month observation period. Of the 14-day-old mice, one was found dead on the 8th day and one on the 9th day, 2 were ill on the 9th day and were found dead on the 10th day, and 4 survived and were discarded on the 21st day. Of the 20-day-old mice, one was found dead on the 5th day and the remaining 7 were obviously ill on the 7th day, showing ruffled fur, hunching, tremors, and ataxia. Four of these animals were killed, and their brains were pooled

and passaged by combined intracerebral-intraperitoneal inoculation into 14- to 15-day-old mice; the remaining 3 animals were killed on the 12th day and their brains similarly inoculated in 15-day-old mice. In both cases the passage mice showed ruffling of the fur, convulsions, tremors, ataxia and paralysis of hind or fore limbs after an incubation period of 4 to 6 days.

Aerobic and anaerobic bacteriologic cultures of infected brains were either sterile or occasionally showed the presence of a few saprophytic bacteria. Since Berkefeld N and W, and Seitz EK filtrates were bacteriologically sterile yet highly pathogenic for mice, the agent was regarded as a virus. For convenience it has been designated the Winkler strain.

In an attempt to identify the agent, guinea pigs and hamsters were inoculated intracerebrally (0.1 ml), intraperitoneally (0.5 ml) or by both routes combined with a 1 percent suspension of brains from the 3rd mouse passage. Three animals were inoculated by each route, all survived without showing any evidence of infection during a 3-week observation period. The lack of pathogenicity for guinea pigs was considered possibly to rule out several viruses, and the failure to produce apparent infection in hamsters to rule out others including the St. Louis virus, to which this species has been reported as susceptible.^{5,6} Nevertheless, on the assumption that newly isolated strains of a virus might fail to produce obvious infection in species normally susceptible to highly passaged laboratory strains of the same virus, neutralization tests were done using the Winkler virus and rabbit or guinea pig immune sera to the Eastern equine encephalomyelitis, Western equine encephalomyelitis, lymphocytic choriomeningitis, Japanese B St. Louis (Hubbard strain) and Winkler viruses and to an unidentified virus isolated from mosquitoes of California by Hammon

⁵ Brown, G. O., Muether, R. O., Mezera, R. A., and LeGier, M. *Proc. Soc. Exp. Biol. and Med.*, 1941, **46**: 601.

⁶ Lennette, E. H. *Proc. Soc. Exp. Biol. and Med.*, 1941, **47**: 178.

TABLE I

Effect of Age of Animal and Route of Inoculation on the Isolation of St Louis Encephalitis Virus in Mice

Route of Inoculation	14 days			Age of mice 21-28 days			6-7 weeks		
	Mortality ratio	% Mort	A S T †	Mort ratio	% Mort	A S T	Mort ratio	% Mort	A S T
I P	20/24	83	11.0 days	5/27	18	18.9 days	3/18	17	19.0 days
I C	16/18	88	9.8	16/26	62	14.9	8/21	38	17.1
I P + I C	13/14	93	11.1	14/21	67	15.9	11/23	48	16.7

* Mortality ratio: the numerator represents the number of mice that died, the denominator the number of mice inoculated.

† A S T: average survival time, computed on the basis of a 21-day observation period, according to the method devised by Kerr and described by Bugher.⁹

and Reeves.⁷ The tests were set up according to the technic previously described.⁸ Undiluted sera were mixed with dilutions of virus and inoculated intraperitoneally in 0.03 ml amounts into mice 14 days of age, a group of 4 mice was used for each serum-virus mixture. The immune serum against the Hubbard strain of St Louis virus neutralized 300,000 LD₅₀ of the Winkler virus. The homologous Winkler immune serum neutralized >300,000 LD₅₀ and the remaining sera neutralized 10 LD₅₀ or less of the virus.

Neutralization tests were then done in the reverse direction, using the Hubbard strain of St Louis virus and Winkler and Hubbard strain immune sera. Both sera neutralized >100,000 LD₅₀ of the known St Louis virus.

The Winkler virus was therefore regarded as a strain of St Louis encephalitis virus. Inasmuch as the patient became ill within 6 to 7 days after his arrival in San Joaquin County, it is not impossible that he may have acquired his infection in Kern County. This is a point of some interest since, as mentioned above, Hammon and Reeves¹ in 1944 had found mosquitoes in Kern County to be infected with St Louis encephalitis virus.

The Winkler virus was subsequently isolated in 2 additional instances in which the pathogenicity of the original human material was tested in mice of different ages. Mice

of 14 days of age, and of 21 to 28 days and 6 to 7 weeks of age were inoculated intracerebrally (0.03 ml), intraperitoneally (0.1 ml), or by both routes, with a 10 percent suspension of human brain tissue, the animals were examined daily during a 4-week observation period to record the number of animals sick or dead.

The results of both experiments are summarized in Table I. As is indicated by the mortality data, the 14-day-old mice were much more highly susceptible to infection by the intraperitoneal route than were the older mice. Similarly, the 14-day-old animals were more susceptible to infection by the intracerebral or combined intracerebral-intraperitoneal routes than were the older animals, the difference in the mortality between the 2-week-old and the 6- to 7-week-old animals is especially striking, and quite different from that observed with a highly passaged laboratory strain of St Louis virus inoculated intracerebrally.¹⁰ The average survival times⁹ also point to the decreasing susceptibility with age to infection by the several routes used. It appears, therefore, that for the isolation of certain viruses, at least, the use of young animals is desirable, and that if older animals are employed somewhat larger numbers should be inoculated to compensate for the lower degree of susceptibility. It would seem, also, that combined inoculation by 2 routes is a desirable procedure to enhance

⁷ Hammon, W. McD., personal communication. The antiserum to this virus was kindly supplied by Dr. Hammon.

⁸ Lennette, E. H., and Koprowski, H., *J. Immunol.*, 1944, **49**, 375.

⁹ Bugher, J. C., *Am. J. Trop. Med.*, 1940, **20**, 809.

¹⁰ Lennette, E. H., and Koprowski, H., *J. Immunol.*, 1944, **49**, 175.

TABLE II
Influence of Age on Susceptibility of Hamsters to Infection with the Winkler Strain

Inoculum	Mortality ratios						
	Age of test hamsters						
	2-3 days	4 days	6-7 days	10-13 days	15-16 days	6-8 weeks	6+ mo†
10% human brain	0/5	0/5	0/4			0/3	
10% 3rd passage mouse brain	9/9		10/10	13/13	16/29	1/12	1/10

* Inoculation was done by the intracerebral route, animals up to 16 days of age were given 0.03 ml, older animals 0.1 ml

† Average weight of animals in this group was 53.8 g
 ‡ " " " " " " " " 154 g

the possibility of infection

As mentioned above, hamsters inoculated with a suspension of the human brain survived without obvious signs of infection. Since hamsters are highly susceptible^{5,6} to infection with laboratory passage strains of the St. Louis virus, the apparent lack of pathogenicity of the Winkler strain for this species was investigated. Hamsters of various ages were inoculated intracerebrally with 10 percent suspensions of the original human brain material or of 3rd mouse passage brains; animals up to 16 days of age received 0.03 ml, the older animals 0.1 ml. The results are brought together in Table II.

As is shown in Table II, hamsters as young as 2 to 3 days of age resisted infection with the original human brain material. After 3 passages in mice, however, the virus proved to be highly lethal for young hamsters but not for the older animals. Table II shows that 3rd mouse passage virus was uniformly lethal for hamsters up to 10 to 13 days of age and that the break in susceptibility occurred at about 15 days of age, since only 16 of 29 animals of 15 to 16 days of age succumbed to the infection. Animals 6 to 8 weeks of age or older were practically insusceptible; only 2 of 22 animals in this group died, one with and one without obvious evidence of illness on the day preceding death.

On the possibility that the local hamster strain might represent one resistant to the St. Louis virus, the Hubbard strain of virus, used in previous studies⁶ on the susceptibility of hamsters to St. Louis virus, was titrated in animals 6 to 8 weeks of age. Serial ten-fold dilutions of a 10 percent suspension of 100th mouse passage virus were prepared in

serum broth and inoculated intracerebrally in 0.1 ml amounts. 2 animals were used for each dilution. The Hubbard strain proved to be uniformly lethal for local hamsters through a dilution of 10^{-7} , the highest tested. The resistance of hamsters older than 15 days to lethal infection with the Winkler virus was therefore considered to be due to the low pathogenicity presumably associated with the low number of passages of this strain, and not to a refractory state peculiar to the strain of hamsters used.

Hammon¹¹ has observed that freshly isolated strains of Western equine encephalomyelitis virus may show a complete lack of pathogenicity for guinea pigs, similar to that described here for the Winkler St. Louis virus in hamsters. Much of the recorded information on the susceptibility of various animal species to a given virus is based on the use of highly passaged laboratory strains. Failure of original source material to induce apparent infection in an animal species used for primary isolation of a virus and generally regarded as susceptible to that virus, or of freshly isolated, low-passage virus to produce overt infections in such species, should therefore be accepted with reservations and not used as an infallible criterion for the elimination or inclusion of certain viruses attendant on attempts at identification.

Summary The virus of St. Louis encephalitis was isolated from a fatal human case of encephalitis in California. This represents the first isolation of this virus from any vertebrate host outside the endemic St. Louis area and the second isolation of this virus in California.

¹¹ Hammon, W. McD., personal communication.

It is considered probable that the patient acquired his infection in Kern County, where Hammon and Reeves have shown the ex-

istence of naturally infected mosquitoes

The pathogenic properties of the virus are described and discussed

15276

Elimination in Human Feces of Infectious Hepatitis Virus Parenterally Introduced *

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Experiments in the transmission of *infectious hepatitis* and *homologous serum jaundice* to human volunteers have revealed certain similarities and differences in the properties and distribution of virus, and route of infection in these 2 conditions. These points have been summarized recently by Neefe *et al*.¹ The etiologic agents of both conditions are filtrable, resistant to a temperature of 56°C for 30 minutes, and transmissible to human volunteers in serial passage.²⁻⁵

In contrast to these similarities are certain differences, namely, (1) the etiologic agent of infectious hepatitis is present in the serum and feces of patients in the acute phase of the naturally occurring or experimentally produced (by feeding) disease.⁶⁻¹¹ On the other

hand attempts to demonstrate the etiologic agent of homologous serum jaundice in the stools of patients with this disease have been unsuccessful.^{1, 11, 12} (2) Infectious hepatitis may be produced in human volunteers by feeding or parenteral inoculation of infectious material.^{2, 6-11, 13} In contrast, while homologous serum jaundice has been produced experimentally by parenteral inoculation of infectious serum,^{3-5, 9, 14} attempts to transmit this condition by feeding similar material have, with one exception, been unsuccessful.^{1, 5, 12} This one exception is an experiment of MacCallum and Bauer⁴ who produced the disease in a human volunteer by feeding infectious serum.

In view of these apparent differences it appeared possible that the *route of inoculation* might affect the distribution of virus and thereby constitute an artificial difference between these 2 conditions in experimental subjects. Failure to recover virus from the stool of patients with homologous serum jaun-

* Representing work done for the Commission on Neurotropic Virus Diseases, Army Epidemiological Board, Preventive Medicine Service, Office of the Surgeon General, United States Army.

Acknowledgment is made of the assistance and cooperation of the following agencies: Selective Service, Camp Operations Division and the Civilian Public Service Unit No. 140.

¹ Neefe, J. R., Stokes, J., Jr., and Gelles, S. C., *Am J Med*, 1945, **210**, 561.

² Havens, W. P., Jr., *Proc Soc Exp Biol and Med*, 1945, **58**, 203.

³ Oliphant, J. W., Gilman, A. G., and Larson, C. L., *Pub Hlth Rep*, 1943, **58**, 1233.

⁴ MacCallum, F. O., and Bauer, D. J., *Lancet*, 1944, **1**, 622.

⁵ Paul, J. R., Havens, W. P., Jr., Sabin, A. B., and Philip, C. B., *J A M A*, 1945, **128**, 911.

⁶ Voegt, H., *Munchen Med Wchenschr*, 1942, **89**, 76 (Abstr.) *Bull Hyg*, 1942, **17**, 331.

⁷ Cameron, J. D. S., *Quart J M*, 1943, **12**, 139.

⁸ MacCallum, F. O., and Bradley, W. H., *Lancet*, 1944, **2**, 238.

⁹ Havens, W. P., Jr., Ward, R., Dull, V. A., and Paul, J. R., *Proc Soc Exp Biol and Med*, 1944, **57**, 206.

¹⁰ Endlin, G. M., and Wilcox, R. R., *Lancet*, 1945, **1**, 212.

¹¹ Neefe, J. R., Stokes, J., Jr., and Rheinhold, J. G., *Am J Med Sc*, 1945, **210**, 29.

¹² Unpublished experiments of the author.

¹³ Neefe, J. R., and Stokes, J., Jr., *J A M A*, 1945, **128**, 1063.

¹⁴ Neefe, J. R., Stokes, J., Jr., Rheinhold, J. G., and Lukens, F. D. W., *J Clin Invest*, 1944, **23**, 536.

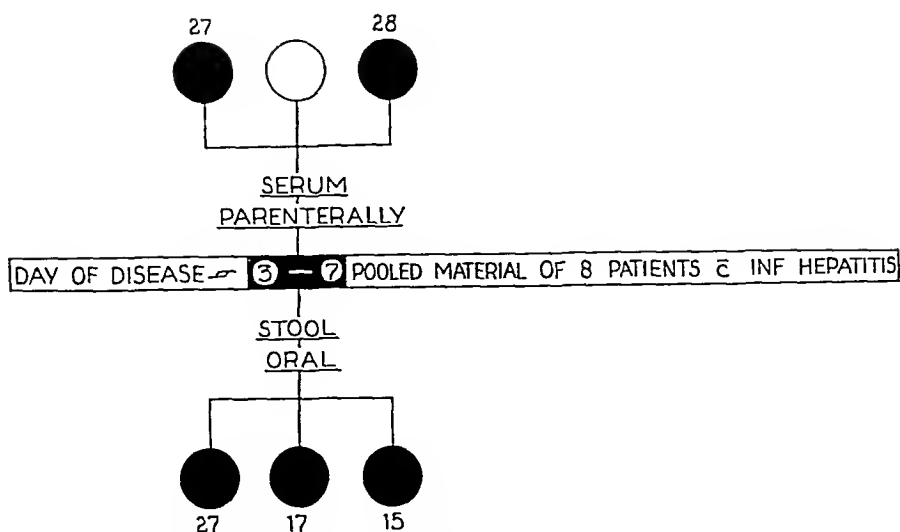


Fig 1

Illustration of results of administration to human volunteers of pools of serum and stool obtained during the acute phase (3-7th day) of 8 patients with infectious hepatitis experimentally induced by parenteral inoculation of infectious material. Open circle indicates a volunteer who was inoculated and failed to contract the disease, black circles indicate volunteers who contracted infectious hepatitis. The figure adjacent to the black circles represents the length of incubation period in days.

dice suggested that more information on the relation between this condition and infectious hepatitis might be gained if a similar situation were found when infectious hepatitis was produced by *parenteral inoculation*.

It is the object of this paper to determine whether a strain of infectious hepatitis virus, introduced *parenterally* into human volunteers, is eliminated in the feces of these same subjects when they contract the disease.

Materials and Methods Virus The strain of infectious hepatitis virus used in this laboratory was originally obtained from the stool of a U S Army soldier (BE) who contracted *epidemic infectious hepatitis* in Sicily in September 1943¹. It has been through 4 passages in human volunteers to date. This agent is filtrable through an L2 Chamberland filter and withstands heating to 56°C for at least 30 minutes². It has produced the disease in 27 out of 40 human volunteers (including this experiment) inoculated parenterally or orally with incubation periods ranging from 15 to 34 days.

Infectious materials employed in this experiment were pooled specimens of serum and of stools obtained during the acute (3-7th

day) stage of disease experimentally induced in 8 volunteers by *parenteral inoculation* of this strain of infectious hepatitis. Stool and serum were always collected from the same patient on the same day. All material was stored at dry-ice box temperature for periods ranging from 3-7 months. Before use equal amounts of the same material from each volunteer were pooled and treated in the following manner: (a) serum was heated to 56°C for 30 minutes in a water bath, (b) stools were ground with sterile alundum and suspended in enough sterile 10/M buffered sodium phosphate to make a 10 percent suspension. This was centrifuged at 1500 r p m for 30 minutes at room temperature to remove coarse particles. The supernate was removed and centrifuged at 6500 r p m for 30 minutes at room temperature. This fairly clear supernate was removed and filtered through a Seitz EK filter at a pH of 7.0. The filtrate was then heated to 56°C for 30 minutes in a water bath. The serum and filtrates of stool were heated to eliminate the possibility of the presence of pathogenic bacteria. Both materials were sterile before administration.

TABLE I

Duration and Height of Fever and Jaundice and Severity of Illness in 5 Human Volunteers with Experimentally Induced Infectious Hepatitis

Volunteer	Inoculum	Source*	Amount	Route	Duration days			Maximum		Severity
					Incubation	Fever	Jaundice	Fever†	Serum Bilirubin mg %	
BS	S	37th day disease (8 patients)	0.5	P	27	13	13	103.8	7.6	++
TR	S	"	0.5	P	28	5	14	101.0	2.5	+
SR	F	"	10	O	27	12	30	104.0	7.0	+++
HD	F	"	10	O	17	5	22	100.5	7.3	++
FW	F	"	10	O	15	10	14	102.5	7.4	++

S = Serum, F = Feces, P = Parenteral, O = Oral

* Cf Fig 1 † Rectal temperatures are recorded

Feces were administered as Seitz filtrates of 10% suspensions

Three volunteers were fed the stool filtrate and 3 were inoculated sub- and intracutaneously with serum. The results are indicated in Fig 1. It is apparent that virus is present in both the stool and serum of patients in the acute-phase of *parenterally induced* infectious hepatitis. Certain clinical data on the 5 volunteers who contracted the disease in this experiment are recorded in Table I.

Summary 1 A strain of infectious hepatitis virus inoculated *parenterally* into 8 human

volunteers was recovered from pools of serum and feces obtained from these same subjects during the acute-phase of their illness. These materials produced infectious hepatitis in 5 out of 6 healthy, human volunteers on re-inoculation.

2 This recovery of virus from the stool of patients with infectious hepatitis induced by *parenteral inoculation* constitutes an apparent difference between this condition and homologous serum jaundice in which the etiologic agent has not been recovered from the stool up to the present time.

15277 P

Transplantability of Induced Granulosa Cell Tumors and of Luteoma in Mice. Secondary Effects of These Growths*

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Granulosa cell tumors can be readily induced in the ovaries of mice by exposing the latter to X-rays.¹⁻³ A single dose of 87r

given over the entire body to 44 mice approximately 6 weeks of age produced tumors in 31 mice. The induced ovarian growths

1936, 28, 54

2 Furth J, and Butterworth, J S, *Am J Cancer*, 1936, 28, 66

3 Geist, S H, Grimes, J H, and Pollack, A D
Am J Obs and Gyn, 1939, 38, 786

* These investigations have been supported by The Anna Fuller Fund, The International Cancer Research Foundation and The Jane Coffin Childs Memorial Fund for Medical Research

1 Furth, J, and Furth, O B, *Am J Cancer*,

TABLE I

	Males		Females		Total	
	Implanted	+	Implanted	+	Implanted	+
F1 mice						
Normal	23	11	21	8	44	19
X-rayed	5	1	20	12	25	13
Gonadectomized	10	8	15	12	25	20
Total	38	20	56	32	94	52
Parental (AK or Rf mice)	25	0	11	0	36	0

were of 3 main histological types (a) tubular adenomas (b) granulosa cell tumors and (c) luteomas. The microscopic characteristics of these tumors have already been described and illustrated.²

Of 21 attempts at transfer of induced ovarian tumors, 13 proved successful. Induced tubular adenomas of 3 mice were successfully transplanted but only one has been carried in serial passages. This is a very slow growing tumor, becoming palpable (about 2 mm in size) after approximately 6 months (Strain VI). Two induced luteomas proved readily transplantable (Strains IX and XI). Cells of luteomata proliferate by mitotic division retaining the morphological characteristics of lutein cells in mice of both sexes. Mice bearing luteoma of Strain IX do not show evidence of hyperestrinization; the adrenal cortices undergo profound atrophy. These mice gained much weight, mainly by excessive deposition of fat in the normal fat depots.

The other transplantable ovarian tumors are granulosa cell growths. Strain I recently described⁴ produces a cavernous dilatation of the liver sinusoids with only occasional secondary parenchymal damage which led to the suggestion that abnormal steroids may have been produced by the tumor and metabolized in the liver.⁴

Granulosa cell carcinoma, Strain III, now to be described, originated in an F1[†] mouse that had been given 175r at an age of 6 weeks and was subsequently painted with 0.5 percent methylcholanthrene dissolved in

benzene (cf⁵). Nine months after the irradiation a tumor felt at the site of the left ovary was removed and fragments of it were implanted into the subcutaneous tissue of this and 10 other F1 mice. When the donor animal died 7 weeks later the transplant measured 2x2x1 cm and the lung contained metastatic ovarian carcinoma.

The results of 9 subsequent transplantation experiments (4 successive passages) are summarized in Table I.

These figures indicate that the range of transmissibility of these ovarian tumor cells resembles closely that of normal cells (cf⁵). At first the tumors grew slowly, becoming palpable only after about 4 months, but later their growth vigor increased and they became palpable within 6 to 8 weeks after transplantation. Metastases occurred in the liver and lungs.

The histological character of the tumor remained unaltered throughout the course of these passages. The tumors are composed of small granulosa-like cells which do not form follicles. They are widely separated by some pale staining material, perhaps their secretion. Necrosis with secondary calcification is common in the tumors and in such areas fibroblast-like cells can be seen which contain alkaline phosphatase granules in abundance and occasionally there is ossification.

There is continued estrus in most spayed or normal female mice bearing large tumors of Strain III, while in male mice the testes and seminal vesicles undergo profound atrophy. The thymus is markedly atrophic.

⁴ Furth, J., and Boon, M. C., *Proc Soc Exp Biol and Med*, 1945, **58**, 112.

[†] Rf x AK hybrid.

⁵ Furth, J. and Boon, M. C., *Science*, 1943, **98**, 138.

in all tumor-bearing mice. In a few mice osteosclerosis of the femur, similar to that produced by estrogenic hormones⁷ is noted. In addition, most, if not all, mice with large transmitted tumors had a cavernous dilatation of the sinusoids of the liver, spleen and adrenals. The weight of the liver was approximately 3 times normal in many mice, accounted for almost exclusively by an increment of blood. Although blood volume determinations have thus far not been made, the observations made suggest a marked rise in blood volume in mice exhibiting the liver change described. The cavernous dilatation of vessels was localized to viscera of the abdomen named. The vessels leading to the grafted tumor were also tremendously distended.

The mouse in which Strain III originated received both X-rays and methylcholanthrene. Extensive experience has shown that the latter alone does not produce ovarian tumors.

⁶ Kaliss, N., and Robertson, T., *Genetics*, 1943, **28**, 78.

⁷ Gardner, W. U., and Pfeiffer, C. A., *Proc Soc Exp Biol and Med*, 1938, **37**, 678.

while X-rays alone have done so in almost as high a percentage of the mice as the combined treatment with X-rays and methylcholanthrene. However, a preliminary tabulation of our data shows that in mice receiving the combined treatment the tumors were larger, appeared sooner, were more readily transmissible and more apt to metastasize than those receiving X-rays only. In the latter, most tumors were of the tubular adenoma type, presumably derived from downgrowth of the germinal epithelium, while in the former, most tumors were of the granulosa or lutein cell types.

Summary Eleven ovarian tumors, induced by X-rays have been transmitted in successive passages. Eight were of the granulosa cell types, 2 were luteomas and one was a tubular adenoma. Histological changes indicate that cells of Strain III secrete estrogens. Mice bearing tumors of this strain also have a cavernous dilatation of the sinusoids of liver, spleen and adrenals. In mice bearing large luteoma of Strain IX there is profound atrophy of the adrenal cortex.

15278

A Method for Detection of Streptothricin in the Presence of Streptomycin

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Streptomycin¹ and streptothricin,² two antibiotic agents having almost identical bacterial spectra,^{3,4} are both produced by members of the actinomycetes, namely, *Actinomyces griseus* and *Actinomyces lavendulae* respectively. Because of this close relationship and

of the still existing difficulty in the identification of the various members of the actinomycetes,⁵ it is possible that strains of the organisms producing these 2 antibiotics might be confused. Such a confusion would be particularly undesirable when it is recognized that streptothricin is many times more toxic than streptomycin and in addition, has a distinct delayed toxicity.^{6,7} Streptomycin,

¹ Schatz, A., Bugie, E., and Waksman, S. A., *Proc Soc Exp Biol and Med*, 1944, **55**, 66.

² Waksman, S. A., and Woodruff, H. B., *Proc Soc Exp Biol and Med*, 1942, **49**, 207.

³ Robinson, H. J., Smith, D. G., and Graessle, O. E., *Proc Soc Exp Biol and Med*, 1944, **57**, 226.

⁴ Robinson, H. J., Graessle, O. E., and Smith, D. G., *Science*, 1944, **99**, 540.

⁵ Waksman, S. A., and Henrici, A. T., *J. Bact.*, 1943, **40**, 337.

⁶ Rake, G., Hamre, D., Kavanaugh, F., Koerber, W. L., and Donovan, R., *Am J Med Sc*, 1945, **210**, 61.

⁷ Robinson, H. J., Graessle, O. E., Gundel, M., and Silber, R. H., in press.

on the other hand, because of its relative non-toxicity and its active inhibition of gram-negative bacteria both *in vitro* and *in vivo*⁸ has aroused a considerable amount of interest in the medical field. Realizing the difficulties which would be encountered if samples of streptomycin were to become contaminated with the more toxic streptothricin, it was considered essential that there be developed a means of detecting small amounts of streptothricin in the presence of large quantities of streptomycin. A method for differentiating between streptomycin and streptothricin has been announced recently.⁹

However, a more simplified microbiological test has been devised which can detect as little as 0.1% of streptothricin present as a contaminant in streptomycin. The procedure is based upon a modification of the Stebbins and Robinson¹⁰ cup-assay for streptomycin. The organism used in the test is a strain of an unidentified gram-negative rod^{*} which was found to be very resistant to streptomycin, but sensitive to streptothricin when tested for sensitivity by means of the agar-streak method. By this technic its growth was found to be uninhibited by 16,000 units[†] of streptomycin per ml, but to be inhibited by 7.5 units of streptothricin per ml. This strain is maintained by daily transfer in broth and a 6-hour culture, which has been incubated at 37°C, is used in the assay in the following manner: pour-plates are made using 10 ml of modified F.D.A. agar¹⁰ which has been seeded

while still fluid so as to contain finally a 10^{-4} dilution of the 6-hour culture. After the agar solidifies, penicillinders are set upon it, and the plates are then ready for use according to the regular cup-assay.

For the establishment of a standard curve from which to estimate the amounts of streptothricin present in the samples of streptomycin a series of solutions are prepared as follows: (1) A stock solution of streptomycin is made in distilled water to contain 2000 units of streptomycin per ml of water. (2) A stock solution of streptothricin is prepared in distilled water to contain 40 units of streptothricin per ml of water. (3) Using solution No. 2 additional solutions are made to contain 20, 16, 8, 4 and 2 units of streptothricin per ml. (4) 0.5 ml quantities of the stock-solution of streptomycin (2000 units per ml) are then combined with 0.5 ml amounts respectively of the 6 solutions of streptothricin (40, 20, 16, 8, 4 and 2 units per ml) to give 6 standard solutions containing 1000 units of streptomycin per ml mixed with streptothricin to the extent of 20, 10, 8, 4, 2 and 1 units per ml. (5) A seventh standard solution consists of 1000 units of streptomycin per ml of water with no added streptothricin. (6) The test samples are made to correspond to the seventh standard solution, namely, to contain 1000 units of streptomycin per ml of distilled water. When prepared the solutions of the test samples together with those of the 7 standards which should always be run at least in duplicate with every new test, are put in the cups for assay against the streptomycin resistant strain. The plates are then incubated at 30°C for 16-18 hours after which they are read for inhibition of growth as shown by the millimeters of zonation produced around the cups. Depending upon the degree of zonation produced, the amount of streptothricin present in the streptomycin can be detected. Table I shows the millimeters of zonation produced by the standard solutions. No sample of streptomycin whether it was of low potency or of almost pure material gave any zonation when used in a concentration of 1000 units

⁸ Jones, D., Metzger, H. J., Schütz, A., and Waksman, S. A. *Science* 1944, **100**, 103.

⁹ Denkewalter, R. G., Cook, M. A. and Tishler, M., *Science*, 1945, **102**, 12.

¹⁰ Stebbins, R. B. and Robinson, H. I. *Proc. Soc. Exp. Biol. and Med.* 1945, **59**, 255.

* This organism was isolated by Dr. Bodinheimer from the trachea of a patient at the College of Physicians and Surgeons, Columbia University, New York City.

† One unit is that quantity of streptomycin which will just inhibit a given strain of *E. coli* (Waksman) in 1 ml of nutrient broth or agar. When measured in terms of antibacterial activity one unit of streptothricin is equivalent to one unit of streptomycin.

TABLE I
 Diameters of Cleared Areas Produced by a Standard of Mixtures of Streptothricin and Streptomycin with Water as the Diluent

Concentration of antibiotic agents				Zonation
1000 units streptomycin per ml only				0 mm
" "	" "	" "	plus 20 units streptothricin per ml	18.0 mm
" "	" "	" "	" " " 10 "	16.5 "
" "	" "	" "	" " " 8 "	15.5 "
" "	" "	" "	" " " 4 "	13.5 "
" "	" "	" "	" " " 2 "	12.0 "
" "	" "	" "	" " " 1 "	8.5 "

of streptomycin per ml. However, concentrations of over 1000 units per ml produced moderate zones, for example 7.8 mm for 5000 units and 10.5 mm for 10,000 units per ml.

This culture has been maintained by daily transfer in this laboratory for a period of 8 weeks without showing any indications of

changing in its response to streptomycin and streptothricin.

With this test one unit of streptothricin can be measured in the presence of 1000 units of streptomycin. Therefore it would be possible to detect as little as 0.1% of streptothricin if it were present as a contaminant in lots of streptomycin.

15279

Biological Conversion of n-Butyl Penicillin into a Chemotherapeutically Active Substance

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Due to its rapid rate of absorption and excretion, it is impossible to maintain satisfactory blood levels of penicillin for prolonged periods after parenteral injection of an aqueous solution of penicillin. These characteristics necessitate either a frequent intermittent or a continuous type of injection. Attempts have been made to prolong the blood levels after parenteral injection of salts of penicillin by decreasing either their rate of absorption¹⁻⁴ or their rate of excretion.^{5,6}

Since the rapid rate of elimination of penicillin following its parenteral injection is due in part to its extreme solubility in aqueous phases, it would appear advantageous to modify the penicillin molecule in such a manner as to form water insoluble derivatives. This goal has been accomplished by the production of various esters of penicillin. Meyer and his co-workers have reported the preparation of a number of such esters including the methyl, ethyl, n-butyl and benzhydryl esters.⁷ All of these compounds were de-

* On leave of absence from the May Institute for Medical Research and the Department of Bacteriology of the College of Medicine, University of Cincinnati, Cincinnati, Ohio.

¹ Fisk, R. T., Foord, A. C., and Alles, G., *Science*, 1945, **101**, 124.

² Parkins, W. M., Wiley, M., Chandy, J., and Zintel, H. A., *Science*, 1945, **101**, 203.

³ Romarsky, M. J., and Rittman, G. E., *Bull*

U. S. Army Med. Dept., 1944, **81**, 43.

⁴ Trumpei, M., and Hutter, A. M., *Science*, 1944, **100**, 432.

⁵ Bevel, K. R., Peters, L., Woodward, R., and Verway, W. F., *J. Pharmacol.*, 1944, **82**, 310.

⁶ Rummelkamp, C. H., and Bradley, S. E., *Proc. Soc. Exp. Biol. and Med.*, 1943, **53**, 30.

⁷ Meyer, K., Hobby, G. L., and Dawson, M. H., *Proc. Soc. Exp. Biol. and Med.*, 1943, **53**, 100.

TABLE I

The Protective Action of Subcutaneous Injections of n-Butyl Penicillin Against Streptococcal Infections in the Mouse

Wt of Ester injected, mg	Dilution of culture in g	No of mice in g	No of mice survived	No of mice died
0	10-6	3	1	2
	10-5	3	0	3
	10-4	3	0	3
0.1	10-6	3	2	1
	10-5	3	2	1
	10-4	3	1	2
0.5	10-6	3	3	0
	10-5	3	3	0
	10-4	3	3	0

Note: Doses of 1.0 mg and 5.0 also gave complete protection

scribed as insoluble in neutral or slightly alkaline buffers but were quite soluble in fat solvents. They were almost devoid of any activity *in vitro* but apparently could be hydrolyzed in alkaline solution to regenerate an active principle, presumably the non-esterified penicillin.⁸

In contrast to their relative inactivity *in vitro*, the ethyl and n-butyl esters were demonstrated to be effective chemotherapeutic agents in mice infected with large doses of virulent hemolytic streptococci.⁷ Thus, single doses of these esters, given either orally or subcutaneously, protected mice against intraperitoneal injections of streptococci. The protective action of methyl penicillin in mice infected with spirochetes has also been demonstrated.⁹ It appeared, therefore, that these esters were hydrolyzed *in vivo* with the slow liberation of active penicillin. Such results suggested the possibility of the successful use of penicillin esters as chemotherapeutic agents in man.

More recently, following the preparation of the benzyl ester of penicillin,¹⁰ reference was made to unpublished results¹¹ of the successful use of this ester in clinical infections and high promise was held forth for

the effectiveness of penicillin esters against infections in man. In contrast to these results, previous work in our own laboratory had indicated that higher mammals were unable to effect the conversion of alkyl penicillin esters into chemotherapeutically active substances. Accordingly, we report a few preliminary experiments obtained with the n-butyl ester in the dog, monkey and man.

Methods and Results Four of the butyl penicillin samples were dark brown viscous oils, one was a yellowish powder. Their *in vitro* activity was tested by dissolving a weighed amount in absolute alcohol and diluting further with broth. Their activity was then assayed against the C203 MV strain of hemolytic streptococcus and all samples showed an activity of 5 to 10 units per mg as opposed to 600 to 1100 units per mg of the original free penicillin from which they had been prepared.

In work with dogs and monkeys, weighed amounts of the ester were dissolved in absolute alcohol and diluted with 4 parts of propylene glycol. The alcohol-glycol solutions were then injected without further sterilization. Preliminary to injection into man, the ester was dissolved in the alcohol-glycol mixture as described above and sterilized by filtration through a small sterile sintered glass filter. The filter was washed with small amounts of absolute alcohol until the washings showed no traces of color. The solution was concentrated in the cold under high vacuum until alcohol was no longer detectable. The residue was then assumed to contain the total amount of ester originally

⁸ Hickey, R. J., *Science*, 1945, **101**, 462.⁹ Richardson, A. P., Walker, H. A., Loeb, P., and Miller, I., *J. Pharm.*, 1945, **85**, 23.¹⁰ Cavallito, C. J., Kirchner, F. K., Miller, L. C., Butler, J. H., Khmek, J. W., Warner, W. F., Sutler, C. M., and Trinter, M. T., *Science*, 1945, **102**, 150.¹¹ Grimble, T. O., Miller, L. C., and Trinter, M. L., *Am. J. Obst. and Gynec.* in press.

TABLE II
Recovery of Free Penicillin from the Urine and Blood after Subcutaneous Injection of n-Butyl Penicillin

Animal	Wt of Ester, mg	Duration of urine collection after inj of Ester, hr	Total free penicillin recovered in urine units	Serum penicillin levels hr after inj							
				1	2	3	4	8	12	24	
Dog	100	48	22	0	*	0	*	0	*	0	
Dog	200	48	31	*	0	*	0	*	0	*	
Monkey	100	36	4	*	*	*	*	*	*	*	
Monkey	200	48	17	*	*	*	*	*	*	*	
Man	200	48	14	0	0	0	0	0	0	0	
Man	200	48	46	0	0	0	0	0	0	0	

* Indicates no determination made

weighed out and was used, in appropriate quantities, for parenteral injection into man

Penicillin assays on blood samples and urine specimens were performed by a serial dilution technic. Since it was believed the urine assays offer a more reliable index of the total absorption of penicillin,¹² such assays were used to indicate the degree of conversion of the ester in the body.

We were able to confirm the results of Meyer, Hobby and Dawson⁷ in regard to the chemotherapeutic activity of n-butyl penicillin in mice. For this purpose, mice weighing approximately 20 g were given intraperitoneal injections of 0.5 ml of a 10^{-4} , 10^{-5} and 10^{-6} dilution of a 15 hour culture of the virulent C203 MV strain of the Group A *Streptococcus*. One-half hour later, the treated animals each received a single subcutaneous injection of the propylene glycol solution of the ester. The results given in Table I indicate that a single injection of 0.5 mg of ester protected all of the mice against 0.5 ml of a 10^{-4} dilution of the culture. Only moderate protection was afforded by 0.1 mg of ester.

No studies on the chemotherapeutic activity of the butyl penicillin were performed on the dog or the monkey, but these animals were used to investigate the pharmacology of this compound and to determine their reaction to large doses subcutaneously.

Two dogs weighing approximately 5 and 7 kg were given subcutaneous injections of 100 mg and 200 mg, respectively, of the ester. The injections apparently produced

a transient moderate local irritation. Observations of both dogs failed to reveal any evidences of a systemic reaction to the injections. Blood and urine assays for free penicillin were performed and the results in Table II indicate failure to produce demonstrable blood levels or more than minute quantities of excreted penicillin.

Similarly, 2 monkeys (*M. mulatta*) weighing approximately 4 kg received 100 and 200 mg each of the butyl penicillin subcutaneously. As was the case in the dogs, the monkeys failed to evidence any reactions to the injections other than a transient mild local irritation. Blood assays were not performed. However, as in the dogs, composite urine samples did not reveal appreciable excretion of free penicillin (Table II).

Inasmuch as the dogs and monkeys appeared to tolerate the injections of penicillin ester, it was considered safe to determine their effect in man. In order to investigate chemotherapeutic activity of the ester, male patients with acute gonorrheal urethritis were chosen since this disease is so sensitive to the activity of penicillin. Two such patients were each given subcutaneous injections of 100 mg of the ester followed 6 hours later by another similar injection. None of the injections produced immediately more than an extremely mild irritation. The following day, however, the site of the injections was swollen, hot and very tender. In addition, there was a moderate febrile reaction accompanied by some malaise. Both the systemic and local reactions had disappeared 48 hours later. Due to the impurity of the preparation it was difficult to assess the

¹² Pedrick, R. F., and Broth-Kahn, R. H., *AAF School of Aviation Medicine Res. Rep.*, 1945, 388-1

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role of the solvent or of the impurities in this moderately toxic reaction. Urine and blood assays were performed and the effect on the disease determined. The results of the assays (Table II) again revealed little or no free penicillin. Clinically, the urethral discharges showed no improvement and smears and cultures continued to reveal the presence of viable gonococci. These observations were continued for 3 days after the injections. If the greater part of the ester had been hydrolyzed, the quantity of free penicillin so liberated might have been expected to produce a demonstrable improvement in the clinical condition. At the end of this period, both patients were treated with 500,000 units of oral penicillin¹³ and their disease responded satisfactorily, thus affording evidence that the infecting strains were not or had not become refractory to penicillin. In order to determine whether the method of preparation of the solution of ester for injection into man had resulted in its inactivation, the same solution was later demonstrated to protect mice against the intraperitoneal injections of 0.5 ml of a 10^{-4} dilution of a 15 hour culture of a virulent hemolytic streptococcus.

Discussion. There would appear to be little doubt that subcutaneous injections of butyl penicillin protect mice against infections by hemolytic streptococci. This protection obviously cannot be attributed to any impurity of unchanged penicillin since 1 mg of the ester contains a maximum of 10 units of activity. Inasmuch as 0.5 mg is sufficient to protect mice, the protective dose contains a maximum of 5 units of free penicillin. Approximately 200 units of free penicillin are required for protection of the mouse against lethal doses of hemolytic streptococci.^{14,15}

The possibility exists either that the esters *per se* exert a chemotherapeutic action or that they, after injection, are converted in the body into active substances. It appears doubtful that the esters themselves are the

actual chemotherapeutic substances since they failed to display any activity against human gonococcal infections and since they are almost devoid of activity *in vitro*. Accordingly, it appears most plausible to assume that, subsequent to absorption, the esters are converted into the active agent. The most probable conversion would undoubtedly be a regeneration of free penicillin. *In vitro* studies¹⁶ have already demonstrated that this reaction occurs. The most reasonable explanation for the mechanism of the activity of the ester in mice would be its constant and somewhat slow conversion, in the body, to free penicillin. If this conversion occurred at an appropriate rate, adequate levels of free penicillin might be maintained in the blood for prolonged periods following a single injection of ester. Once the hydrolysis had occurred the resulting penicillin would be expected to be excreted in the urine at its normal rate.

On the other hand, following subcutaneous injections of the ester in the dog, the monkey and in man, the observations of this study indicate the inability to recover appreciable quantities of penicillin in either the blood or the urine. It would seem, therefore, that the tissues of these mammals are unable to effect the conversion of the ester into free penicillin at an appreciable rate.

In general, the velocity of hydrolysis of aliphatic esters by tissues varies inversely with the molecular weight of the esterifying alcohol.¹⁷ It was therefore believed that efforts should be directed towards investigation of simpler esters. However, Richardson *et al*¹⁸ report results with both the methyl and benzyl esters of penicillin comparable to our own findings with the *n*-butyl ester. In view of the fact that the benzyl radical behaves as an aliphatic derivative, it would have been somewhat surprising to learn that tissues unable to hydrolyze methyl and butyl penicillin could effect the hydrolysis of the

¹³ Broth Kahn, R. H., *AAF School of Aviation Medicine Res. Rep.*, 1945, 4041.

¹⁴ Hobbs, G. L., Meyer, K. and Claffie, D., *Proc. Soc. Exp. Biol. and Med.*, 1942, 50, 285.

¹⁵ Robinson, H. J., *J. Pharm.*, 1943, 77, 70.

¹⁶ Broth Kahn, R. H., and Smith, P. K., unpublished observations.

¹⁷ Hoss, G. M., *Arch. Path.*, 1938, 26, 1183.

¹⁸ Richardson, A. P., Walker, H. A., Miller, I., and Hinson, R., *Proc. Soc. Exp. Biol. and Med.*, 1947, 60, 272.

higher benzyl homologue

Summary and Conclusions 1 A subcutaneous injection of butyl penicillin conferred marked protection against hemolytic streptococcal infections in the mouse 2 Subcutaneous injections of 200 mg exerted no appreciable effect on gonococcal infections in 2 men 3 Following subcutaneous injections of butyl penicillin into the dog, the monkey, and man, appreciable quantities of penicillin were not recovered in the blood or urine 4 The subcutaneous injection of from 100 to 200 mg of butyl penicillin produced no

evidences of marked toxicity in the dog or the monkey Two men reacted with evidences of moderate toxicity 5 The tissues of the mouse can convert the chemotherapeutically inactive butyl penicillin into a chemotherapeutically active substance, presumably penicillin The tissues of man, the monkey, and the dog cannot effect this conversion at an appreciable rate

Dr Karl Meyer kindly furnished us with the butyl penicillin used in this study and supplied the data concerning the *in vitro* activity against the streptococcus

15280

Influence of Iron Salts on the Toxicity of Lead

LEON A HEPPEL AND ARTHUR KORNBERG (Introduced by W F von Oettingen)

From the Industrial Hygiene Research Laboratory, National Institute of Health, Bethesda, Md

The relationship between the toxicity of lead and the dietary level of other elements has been studied for many years The extensive literature has been reviewed by Cantarow and Trumper¹ Calcium and phosphate have been especially implicated in modifying the effects of oral administration of lead salts The influence of iron compounds on the development of lead poisoning has received little study Our attention was directed to this problem during the course of work on blood formation in various toxic states In the present study it was found that iron salts prevented the weight depression and anemia in rats fed diets containing lead acetate

Experimental Albino rats of Wistar and Osborne and Mendel strains were fed the experimental diets at weaning Feeding was *ad libitum* except in Exp 2 The animals were weighed at intervals of 4 to 7 days Tail blood was used for micro-determinations of the hematocrit (Van Allen) and for hemoglobin determinations by the oxyhemoglobin

method of Sanford *et al*² Blood smears were fixed in absolute methanol and stained for 1½ hours with dilute giemsa solution buffered to pH 7.2 The total number of basophilic red cells per 1000 red cells were counted with the aid of a Whipple disc

Effects of ferric citrate and ferrous sulfate In a preliminary study with semipurified diets it was found that supplements of ferric citrate and copper sulfate protected against the depression in weight gain and anemia of lead poisoning Subsequent tests showed that ferric citrate alone was equally effective

Table I shows the results of 2 experiments in which male weanling rats were used*

² Sanford A H, Sheard, C, and Osterberg, A E, *Am J Clin Path*, 1933, 3, 405

¹ Cantarow, A, and Trumper, M, *Lead Poisoning*, The Williams and Wilkins Co, Baltimore, 1944

* The percentage composition of the basal diet for these and all remaining experiments was as follows purified casein (Smaco) 30.0, hydrogenated cottonseed oil (Crisco) 10.0, USP cod liver oil 5.0, choline chloride 0.2, salt mixture 1.9, cane sugar 52.9 In addition, the vitamin supplement (in mg per kilo) was thiamine hydrochloride 10, nicotinic acid 40, pyridoxine hydrochloride 10, calcium pantothenate 40, and riboflavin 20 Each rat received a weekly oral supplement of 3 mg of

TABLE I
Effect of Ferric Citrate on the Toxicity of Lead

	Added to 100 g diet		No of rats	Mean wt g	Mean hemoglobin [†] g/100 cc
	Lead* g	Ferric citrate g			
Exp 1	—	—	12	139 (117 196)	15.1
	0.03*	—	8	106 (85 144)	9.9
	0.03*	1.13	8	148 (111 170)	16.0
Exp 2	—	—	10	149 (123 187)	16.6
	0.03*	—	10	126 (85 156)	12.1
	0.03*	1.13	10	141 (115 180)	14.8
	0.03*	1.47	10	142 (112 172)	15.5

* After 31 days on test, the lead content of the diets was increased to 0.09%

† After 27 days in Experiment 1 and 56 days in Experiment 2

‡ Determinations done after 66 days in Experiment 1 and after 75 days in Experiment 2

TABLE II
Effect of Ferric Citrate and Ferrous Sulfate on the Toxicity of Lead

Added to 100 g basal diet	Mean wt after 51 days g	Mean hemoglobin after 48 days g/100 cc
(1)	213.4	15.8
(2) 0.09 g Pb (as Lead Acetate)	81.5	10.1
(3) 0.09 g Pb + 1.13 g Fe Citrate	168.0	15.3
(4) 0.09 g Pb + 0.25 g Fe Citrate (0.84 mmols)	130.4	12.9
(5) 0.09 g Pb + 0.2 g FeSO ₄ (1.32 mmols)	133.3	15.5

In Exp 1, the differences between the mean weights for the rats on basal diet with lead and the other two groups were examined by Fischer's *t*-technique⁴ and found to be significant with a *P* value below 0.01. With the pair-feeding technic (Exp 2) differences

α-tocopherol in 0.03 cc ethyl laurate. The salt mixture (in g) was composed of: NaCl, 138; CaCO₃, 200; MgCO₃, 50; KH₂PO₄, 424; MgSO₄·7H₂O, 65.6; KCl, 224; MnSO₄·H₂O, 0.784; NaF, 2.0; KI, 0.16; Al₂(SO₄)₃·K₂SO₄·24H₂O, 0.622; CuSO₄·5H₂O, 11.95; Ferric Citrate, 16.55. This salt mixture was designed to make a low calcium high phosphorus ratio in the completed diet. According to the work of Grant *et al.*⁵ such a diet favors the deposition of lead in bone, liver and kidney. Samples of the ferric citrate used were analyzed and found to contain 18.1% of iron.

⁴ Grant R. L., Calvery, H. O., Laug E. P. and Morris H. I. *J. Pharm. and Exp. Therap.* 1938 **64**, 446.

⁵ Fischer R. A. *Statistical Methods for Research Workers*. Oliver and Boyd London, 1936.

in weight gains were small enough to be disregarded. This has been observed before among lead poisoned rats.⁵ But a statistically significant anemia, preventable by generous additions of ferric citrate, was obtained.

In Exp 3 (Table II), 50 male weanling rats were divided into 5 groups equal with respect to average weight and litter distribution. It was found that an addition of 0.25 g of ferric citrate was less effective than 1.13 g in opposing the toxicity of lead. Ferrous sulfate also had some protective action. The differences in average weight gains and hemoglobin concentrations between the group given basal diet containing lead and all of the other groups were analyzed statistically and found to be significant.

Ineffectiveness of sodium citrate Experiments were carried out to determine whether

⁶ Bernstein, H. D., and Grand J. A., *J. Pharm. and Exp. Therap.* 1942, **74**, 18.

TABLE III Effect of Ferric Citrate and Sodium Citrate on Depression of Growth Rate Anemia and Red Cell Polychromatism of Lead Poisoning*

Group	Added to 100 g basal diet				Avg wt g		Hemoglobin		Hematocrit		Polychromatized cells per 1000 b c after 62 days
	Lead g	Ferric citrate g	Sodium citrate g	g	Days on diet		g/100 cc		%		range
					0	25	51	65	26	61	
A	—	—	—	—	43.2	137.3	230.3	239.4	14.2	16.1	38 (26)
B	0.09	—	—	—	40.9	89.7	128.2	143.2	13.1	10.5	49.0 (12.151)
C	0.09	1.13	—	—	39.0	127.1	192.3	203.1	14.7	16.4	82 (0.123)
D	0.09	—	1.15	—	41.3	92.2	128.6	137.1	12.5	10.9	62.6 (10.180)

Significance of Differences Between Means†

Groups compared (first term greater than second term)

Weight gain, 51 days Hemoglobin, 61 days	A B		A C		A D		C B		B D		C D	
	P<0.001	P<0.001	P<0.02	—	P<0.001	P<0.001	P<0.001	P<0.001	P>0.3	—	P>0.6	P<0.001

* Each group contained 10 rats. One rat in group A died after 24 days on experiment and one rat in group B died after 57 days on experiment. † These P values indicate that there was no statistical significance between the weight gains or hemoglobin values of the rats receiving lead alone and those of rats getting sodium citrate added to the lead salt. The non-leaded rats had hemoglobin levels not significantly different from those on basal diet. All other differences between groups were significant. Weight gains for a period of 51 days were included because this covered the phase of most rapid growth for the control rats.

the citrate radicle in ferric citrate had any protective effects. It was first ascertained that sodium citrate could be fed to weanling rats at a level of 1.15 g added to 100 g of diet without depressing the rate of growth. This was equivalent to 1.13 g of ferric citrate.

In a preliminary experiment it was found that sodium citrate did not protect against the growth depressant effect of lead. Young rats were fed the basal diet, the leaded basal diet or the leaded basal diet with 1.15 g of sodium citrate added to every 100 g of diet. In one test 5 rats were placed in each group and in a second test there were 6 rats in each group. In both tests it was found that after 30 days the mean weight of the sodium citrate treated rats was approximately 10 g less than that of the rats receiving only lead.

In the next experiment 40 male weanling rats of the Wistar strain were divided into 4 groups equal with respect to litter distribution. The diets and experimental results are shown in Table III.

Discussion In these experiments iron salts interfered in some way with the toxicity of lead. This was deduced from the fact that the depression of growth rate, anemia and red cell polychromasia, were reduced or absent in rats given supplements of ferric citrate or ferrous sulfate.

The anemia of the leaded rats, although prevented by supplements of ferric citrate, was not related to an inadequate iron content of the basal diet. The rats on the unleaded basal diet did not become anemic even when pair fed against those receiving lead.

It may be of interest to compare the content of ferric citrate in our protective diets with that obtained by one of the better known salt mixtures as ordinarily used. The Mc-

Collum-Davis salt mixture 185,⁶ when fed at a 5% level contributes 0.16% of ferric citrate to the diet. In the present study a supplement of approximately 0.25% of ferric citrate, did not bring about normal hemoglobin concentrations in leaded animals, although it had some beneficial effect. A level of 1.13% prevented the anemia entirely.

It is of interest that Miyasaki⁷ found that a colloidal solution of 2% dialyzed ferric oxide reduced the absorption of lead in mice although a suspension of ferric hydroxide was ineffective. The mechanism of action of iron salts in counteracting the toxicity of orally administered lead in these studies has not been established. However, experiments in progress indicate a much lower lead content of tissues of rats whose diet contained supplements of iron citrate. A possible explanation for these findings would be an interference by iron citrate with the absorption of lead. Further studies including the use of radioactive lead (radium D) for tracing the absorption of lead in the presence of iron compounds are planned. The possibility of an interference with the absorption of iron by lead also merits investigation.

Summary Ferric citrate as 1.12% of the diet protected young rats against the weight loss, anemia and red cell polychromasia of lead poisoning. One-fourth of this amount was less effective. About 0.2% of ferrous sulfate had a protective action. Sodium citrate was ineffective.

⁶ McCollum E. V. and Davis, M., *J. Biol. Chem.*, 1918, 33, 55.

⁷ Miyasaki, S. *Arch. Exp. Path. u. Pharmacol.*, 1930, 150 (1/2), 39.

Miss Virginia T. Porterfield, Mrs. Evelyn G. Perke and Miss D. Louise Odor assisted with the experiments and helped to perform the statistical analyses.

Influence of Certain Substances on Activity of Streptomycin I Modifications in Test Medium

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It was previously shown¹ that the amount of streptothricin required to inhibit the growth of the test organism, *Klebsiella pneumoniae*, in 1% tryptone broth varied with the lot of tryptone employed in preparing the broth. To this extent we had found that streptomycin behaves in a like fashion. Wallace *et al.*² have reported that suspensions of *Eberthella typhosa* showed marked drops in viable cell counts when exposed to streptomycin in the presence of "nutrient broth" and "nutrient broth diluted with an equal amount of water," whereas in brain heart infusion broth the same concentration of streptomycin had no apparent effect. *Staphylococcus aureus* suspensions under similar conditions showed marked drops in viable cell counts in all 3 media during the first 6 hours of exposure to streptomycin, but grew out heavily in brain heart infusion broth during the interval between the 6th and 9th hours of exposure.

The present paper deals with the growth-inhibiting activity of streptomycin in (a) a culture medium containing only tryptone and water or tryptone dextrose and water, and (b) an enriched medium containing sodium thioglycollate.

A. The Activity of Streptomycin in Culture Media Containing Varying Amounts of Tryptone and Dextrose. In the paper already mentioned¹ it was shown that when streptothricin was added to 2 ml of 6-hour broth cultures of *K. pneumoniae* diluted 10⁻⁶ in broth consisting of 1% tryptone (or tryptose) and water, from 0.08 to >0.16 units of streptothricin per ml of broth were required

to cause complete inhibition of growth, as observed after 17 hours incubation at 37°C. The minimum inhibiting concentration of streptothricin varied with the lot of tryptone or tryptose used. As indicated above, the action of streptomycin on *K. pneumoniae* was similarly affected by the various lots of tryptone. In the present paper it will be shown that even when a single lot of tryptone is used for preparing test media, the minimum inhibiting concentration of streptomycin will vary considerably with the concentration of tryptone used. Further, if dextrose is also added to the medium, the minimum inhibiting concentration will again be increased.

Procedure. Media were prepared containing from 0.5 to 1.0% tryptone (from a given lot) to which were added from 0 to 3% dextrose. A 6-hour culture of *K. pneumoniae* was diluted 10⁻⁶ in each of these media. The diluted cultures were dispensed into tubes in 2 ml volumes to which were then added increasing amounts of a standard streptomycin solution containing 2.0 units/ml according to the assay procedure referred to above.¹ The minimum inhibiting concentration (MIC) of streptomycin in each medium determined in this fashion is shown in Table I and Fig. 1.

Thus it is seen that as the concentration of tryptone rises in the test medium, the minimum inhibiting concentration of streptomycin increases. To a lesser extent addition of dextrose to the medium also raises the MIC, and interestingly has a more marked effect when added to 1.0% tryptone than to 0.50% tryptone. The addition of increasing amounts of dextrose to tryptone broth causes decreasing end pH values in the broth on autoclaving. Waksman and Schatz³ have shown that lowering pH of agar lowers the

¹ Donovan, R., Hume, D., Kavanagh, F., and Rake, G., *J. Bact.*, 1945, **50**, 623.

² Wallace, G. I., Rhymer, I., Gibson, O., and Shattuck, M., *Proc. Soc. Exp. Biol. and Med.*, 1945, **60**, 127.

³ Waksman, S. A., and Schatz, A., *J. Am. Pharmaceut. Assoc.*, 1945, **34**, 273.

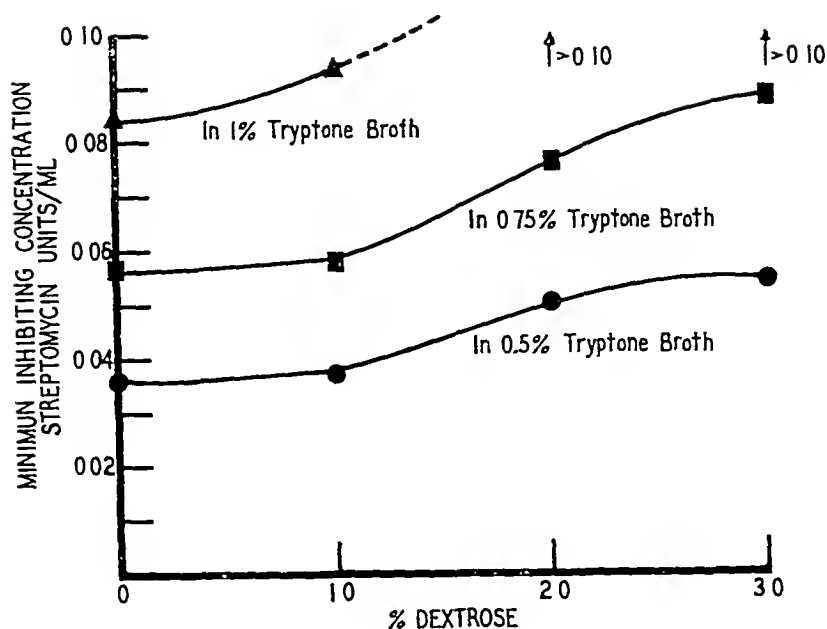


FIGURE 1

Effect of Dextrose and Tryptone in Media on Minimum Inhibiting Concentration of Streptomycin for *K. pneumoniae*

activity of streptomycin in the cup-plate test. We have observed similar effects in broth. Thus the interference caused by dextrose may be due in part to the effect on pH. However this does not explain the finding that dextrose causes greater interference in broth containing 1.0% and 0.75% tryptone than in broth containing 0.5% tryptone since the pH drop with increasing dextrose was identical in all 3 concentrations of tryptone.

B. The Activity of Streptomycin in an Enriched Medium Containing Sodium Thioglycollate Denkewalter, Cook and Tishler³ have reported that streptomycin and streptothricin are 'not inactivated to any signifi-

cant extent by thioglycollic acid.' Their data however, shows that where a phosphate buffer control of streptomycin assayed at 49 units/ml control solutions containing in addition 0.50, 2.50 and 3.0 mg respectively of thioglycollic acid per ml, assayed at 31, 39 and 26 units/ml respectively. Similar results were obtained with streptothricin.

In the present studies we have compared the activity of streptomycin in broth consisting of 0.75% tryptone with that in thioglycollate broth containing the following ingredients:[†]

Cystim	0.75 g
NaCl	2.5 "
Dextrose	5.0 "
Agar	0.75 "
Yeast Extract	5.0 "
Pancreatic digest of casein	15.0 "
Sodium thioglycollate	0.5 "
Resazurin (0.1%)	1 ml
Water to make 1000 ml	

³ At the time that this paper was in proof Bondi, Dietz and Spaulding⁴ published similar findings on the effects of sodium thioglycollate and other reducing substances on the action of streptomycin.

² Denkewalter, R., Cook, M. A. and Tishler, M. *Science* 1945 102, 12.

⁴ Bondi, A. Jr., Dietz, C. A. and Spaulding, E. H. *Science* 1946 103, 399.

[†] Described in a circular from the National Institute of Health (January 15 1945) revising the culture medium required for sterility testing of biologics.

TABLE I
Effect of Tryptone and Dextrose on Minimum Inhibiting Concentration of Streptomycin

% Tryptone	% Dextrose			
	0	10	20	30
	MIC* units/ml			
0.50	0.036	0.037	0.050	0.054
0.75	0.056	0.058	0.076	0.088
1.0	0.084	0.093	>0.10	>0.10

* Minimum inhibiting concentration of streptomycin

TABLE II
Comparison of Minimum Inhibiting Concentrations of Streptomycin in Tryptone and Thioglycollate Broths

MIC in tryptone broth u/ml*	MIC in thioglycollate broth			
	Broth No 1†		Broth No 2‡	
	Age of broth§ days	MIC u/ml	Age of broth§ days	MIC u/ml
0.055	3	1.48	1	>2.98
0.053	6	1.37	2	2.41
0.051	7	1.25	3	2.81
0.057	15	1.04	7	1.52
0.059	16	0.99	8	1.35
0.051	23	1.06		
0.045	24	0.69		
0.055	42	0.63		
0.056	43	0.55		

* Units per ml

† Prepared in this laboratory

‡ Prepared from dehydrated broth obtained from Baltimore Biological Laboratory, Baltimore, Md

§ Age of broth indicates the interval between the time of preparation of the broth and the time when the broth was used. During this interval the flasks of broth remained at room temperature

The media were sterilized by autoclaving at 15 pounds pressure for 20 minutes. After sterilization the pH of both types of media was 7.0-7.2.

A single lot of tryptone was used throughout for preparing the tryptone broth. Two thioglycollate broths, containing the given ingredients, were employed. One was prepared in this laboratory while a second was obtained in a dehydrated form from an outside source. The minimum inhibiting concentrations of streptomycin per ml of test broth are shown in Table II. For the tests a 6-hour culture of *K. pneumoniae* diluted 10^{-6} in the given broth was used.

As is shown in Table II, approximately 27 times more streptomycin was required to inhibit the growth of *K. pneumoniae* in thioglycollate broth No. 1, 3 days after the broth was prepared, than in 0.75% tryptone broth. Forty days later the ratio of MIC values in

these 2 broths had dropped to 10:1. In thioglycollate broth No. 2, when used one day after preparation, the minimum inhibiting concentration was 54 times greater than that in tryptone broth. This ratio decreased to about 24.5:1 after the thioglycollate broth had stood for 8 days at room temperature. It was further found that in a broth containing the same ingredients as thioglycollate broth No. 1 in Table II, except that sodium glycollate was substituted for sodium thioglycollate, the minimum inhibiting concentration of streptomycin was about 10 times greater than that in 0.75% tryptone broth. This compares to thioglycollate broth No. 1 after it had stood at room temperature for 43 days.

Thus it is seen that the incorporation of sodium thioglycollate in a culture medium greatly interferes with the activity of streptomycin. Since sodium thioglycollate is readily

oxidized, this interference with streptomycin activity gradually decreases as the broth stands. What may be called the baseline of interference of the broth, which is reached after some 40 days of standing in one thioglycollate medium we have used, is similar to the interference caused by the same medium containing sodium glycollate instead of sodium thioglycollate.

It is interesting that the action of streptothricin is affected to a similar or perhaps even greater extent in thioglycollate broth. The minimum inhibiting concentration of a streptothricin preparation (320 units/mg) which was tested in thioglycollate broth No. 1 (7 days after preparation) was 2.65 units/ml as compared to 0.053 units/ml in 0.75% tryptone broth—a ratio of 50:1.

It perhaps should be pointed out that the concentration of sodium thioglycollate in the media tested was 0.5 mg per ml while the concentrations said by Denkewalter, *et al.*³ to cause no appreciable destruction of streptomycin ranged from 0.50 to 3.0 mg per ml. Hence remarkable interference with streptomycin activity may occur without destruction of the streptomycin. Since thioglycollates reduce the oxidation-reduction potential of a culture medium, could this interference

indicate that streptomycin interferes more with aerobic than anaerobic metabolism?

Summary Under standard test conditions, raising the concentration of tryptone from 0.50% to 1.0%, in a medium containing only tryptone and water, raised the minimum inhibiting concentration of streptomycin from 0.036 units/ml to 0.084 units/ml. The further addition of glucose to the medium increased the minimum inhibiting concentration to a lesser, but nevertheless, definite degree.

The minimum inhibiting concentration of streptomycin in an enriched broth containing sodium glycollate was about 10 times greater than that in 0.75% tryptone. The substitution of sodium thioglycollate for sodium glycollate further greatly increased the MIC. This additional interference by sodium thioglycollate was observed to decrease as the broth aged (and hence as the thioglycollate was oxidized). Since thioglycollate *per se* is said to cause no significant destruction of streptomycin, it is proposed that its interfering action may be due to its role in reducing the oxidation-reduction potential of the medium and that perhaps streptomycin interferes more with the aerobic than anaerobic metabolism of the test organism.

15282

Metabolism of Glycine by the Completely Isolated Mammalian Heart Investigated with Carboxyl-Labeled Glycine *

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When acetate containing heavy carbon, C^{13} , in the carboxyl position is administered to the completely isolated, working mammal-

ian heart the isotope appears in the respiratory CO_2 rapidly and in large amounts indicating that the 2-carbon chain is readily broken and converted to CO_2 , in amounts approximating 20-30% of the total CO_2 output of the heart.¹ In the present experiments glycine labeled with an excess of C^{13} in the carboxyl carbon, was injected into the blood perfusing the

* Assistance in the preparation of part of these materials was furnished by the personnel of the Work Projects Administration, Official Project No. 165-1-71-440, Subproject No. 392.

† Present address: Illinois Neuropsychiatric Institute, University of Illinois College of Medicine, Chicago, Ill.

¹ Lorber V., Lifson N., Wood H. G. and Barcroft, J. *Am. J. Physiol.* 1946 **145**, 557.

TABLE I
Isotopic Content of Respiratory CO₂ Following Administration of Labeled Glycine to the Isolated Cat Heart

Exp No	Mg of glycine given	CO ₂ collection period in min	mM of carbon collected as respiratory CO ₂	% C ¹³ in the respiratory CO ₂ †
1	50	15	0.38	1.07‡
		15	0.53	1.09
		15	0.65	1.10
		17	0.46	1.07
		15	0.41	—
		30	1.17	1.10
2	100	30	1.70	1.11‡
		30	1.68	1.07
		30	1.52	1.12
		27	0.70	1.13
3	125	30	1.46	1.08‡
		30	1.42	1.09
		30	1.31	1.12
		34	1.13	1.11

* The glycine was given at the beginning of the second collection period in each experiment
Moles C¹³

$$\dagger \% \text{C}^{13} = \frac{\text{Moles C}^{13}}{\text{Moles C}^{12} + \text{Moles C}^{13}} \times 100$$

‡ Control value prior to injection of glycine is 1.09% Normal C¹³ content of C from mineral sources

isolated cat heart in order to determine whether this compound, the amino derivative of acetic acid, is similarly metabolized by cardiac muscle

Methods The experiments were performed on cat hearts, of 6 to 7 g weight, isolated in a manner previously described.² Respiratory carbon dioxide was collected by passing the ventilating gas through absorption bottles containing carbonate-free sodium hydroxide. Total circulating blood volume was 50 to 60 cc.

Total respiratory carbon dioxide was determined by analysis of the absorption alkali by the Van Slyke manometric method. The C¹³ content of the carbon dioxide for each collection period was measured in the mass spectrometer.³

After isolation of the heart was completed, a preliminary control period of 15 to 30 minutes was permitted to elapse, during which time the respiratory carbon dioxide was collected. Following this period, 50 to 125 mg of labeled glycine, dissolved in 0.6 ml of Ringer solution, was injected into the blood,

and the carbon dioxide collection was continued, fresh absorption bottles being introduced at 15 to 30 minute intervals. The glycine used was synthesized⁴ with 7.59% C¹³ in the carboxyl position.

Results The results are presented in Table I. A small increase in the % C¹³ in the respiratory CO₂ above the initial control values is noted in each experiment. The irregularities occurring in Exps 1 and 2, i.e., the low value found in the fourth collection period in 1, and the high value found in the control period in 2, cannot be accounted for, but difficulties in the operation of the mass spectrometer occurring at the time may have been in part responsible. Because of these irrational values, the figures are to be regarded with some reserve. Although if taken at face value the results indicate the breakdown of minute amounts of glycine (about 1 mg) by the heart, the important conclusion to be derived from the data is that the degradation of glycine, under the conditions of our experiments, does not serve as an appreciable source of energy for the heart muscle. This is contrary to the

² Lorber V, *Am Heart J*, 1942, **23**, 37

³ Nier A O *Rev Scient Instruments*, 1940, **2**,

⁴ Olsen, N S, Hemingway, A, and Nier, A O *J Biol Chem*, 1943, **148**, 611

expectation that the high concentrations of glycine maintained and the low blood carbohydrate values obtaining after $1\frac{1}{2}$ to 2 hours (glucose, 14 mg% at the close of Exp 2) in the usual experiment of this type, should have favored the metabolism of glycine. Again, with the present results taken at face value, the glycine may be calculated to have yielded only around 0.5% of the total CO_2 output of the heart (on the assumption that both carbons of the molecule have gone to CO_2) as compared with values of 20-30% from acetate,¹ administered in concentrations of the same order of magni-

tude as those of glycine

Summary Glycine, labeled with heavy carbon in the carboxyl position, was administered to the completely isolated working, cat heart. Isotopic analysis of the collected respiratory CO_2 indicated that if splitting of the 2-carbon chain of glycine with conversion of the carbon to CO_2 was accomplished by the heart muscle, it occurred only to a slight extent.

The authors wish to thank Mr. Charles Stevens, formerly of the Department of Physics, who made the measurements with the mass spectrometer.

15283 P

Blood and Tissue Chemical Studies in Fowl*

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Normal blood and tissue levels of the usual organic constituents with especial reference to total cholesterol and cholesterol esters and other lipids, were determined as a basis for projected fat metabolism studies. Domestic fowl was the chosen experimental animal because of the known high normal cholesterol production, especially of certified 260 to 320 eggs per year R.O.P. sired hens. Laying hens, after the sixth month, have been found to have a subintimal atherosclerosis of the aorta and coronary arteries, quite similar microscopically to the human type in half the specimens examined by Dauber.¹ As the age increases, the frequency of gross lesions has been noted to increase.

We have sacrificed 26 barred rocks fed on a standard growing mash (Purina) at ages

from 11 weeks through 22 weeks and 26 hens of various standard breeds that had been on a standard laying mash, (Purina). Blood was collected from the severed neck vessels; part was ovalated and part was allowed to clot and the serum expressed. The aorta, heart, and in later studies about 0.25 g of liver were removed. The gross adipose tissue was dissected off and approximately 0.25 g of cleaned aorta and lean heart muscle and liver were weighed accurately, minced carefully, extracted for 12 hours with 3 to 1 parts of alcohol and ether.

The blood serum was analyzed by Folin's microchemical methods for glucose,² non-protein nitrogen,³ and Leiboff and Kahn's method for urea nitrogen.⁴ Bloor's original method for cholesterol⁵ and Bloor and Knudson's digitonin method⁶ for cholesterol esters

*Supported by a grant from the Medical Research Department, Winthrop Chemical Co. Choline Chloride was generously supplied by Merck & Co.

The generous cooperation of C. W. Carter of the Texas Agricultural Experiment Station, is gratefully acknowledged.

¹Dauber, D. V. *Arch. Path.* 1944, **38**, 46.

²Folin, O. *J. Biol. Chem.*, 1929, **82**, 92.

Folin and Wu. *J. Biol. Chem.*, 1919, **38**, 81.

³Leiboff, S. L. and Kahn, B. S., *J. Biol. Chem.* 1929, **83**, 347.

⁴Bloor W. R. *J. Biol. Chem.* 1916, **24**, 227.

⁶Bloor W. R. and Knudson, A. *J. Biol. Chem.* 1916, **27**, 107.

TABLE I
Blood Chemical Levels in Normal Fowl

Controls	NPX mg/ 100 ml	Urea mg/ 100 ml	Glucose mg/ 100 ml	Cholesterol mg/ 100 ml	Cholesterol mg/250 g of aorta	Serum protein g/100 ml	Sex and age
Controls (Y) (26) S D	38.5 ±4.7	19.6 ±2.6	171.6 ±23.6	217.4 ±25.0	172.7 ±33.2	4.03 ±0.5	Young B R 11-22 wk old hens
Controls (A) (26) S D	36.2 ±6.4	19.1 ±3.0	177 ±48.4	248 ±37	230 ±43.2	6.1 ±1.3	Various breeds

TABLE II
Blood and Tissue Chemical Levels in Normal Fowl

	Blood cholesterol total mg/100 ml	Esters mg/100 ml	Phospholipids mg/100 ml	Tissue cholesterol, total/esters		
				Aorta mg/250 mg	Heart mg/250 mg	Liver mg/250 mg
Controls (B) (6) S D	232 ±21	153 ±5.6		249/ ±39 T/E	271/ ±49 T/E	337/ ±54 T/E
Controls (C) (26) (4) S D	271 ±37	179 ±19	9.6 2.8	230/176 ±45/±20	288/234 ±86/±19	342/240 ±62/±19

T total, E esters

King's method⁷ for total and inorganic phosphorus and the CuSO_4 falling drop method⁸ for calculating the total serum protein levels.

The ether alcohol extracts of aortic arches, hearts and livers were quantitatively analyzed for content of cholesterol, cholesterol esters and the amount in exactly 0.25 g of each tissue was calculated.

The 26 young barred rocks showed normal levels for the usual blood constituents (Table I). The cholesterol levels were relatively low in both sexes from the 11th to 14th weeks and then started to rise especially in the

pullets seemingly dropped off some at the 16th week as minute egg yolks appeared and then rose slowly. The cholesterol levels in the aortae seemed to follow the blood levels. The blood protein levels were lower than for mammals.

Among the 26 older hens, all showed normal values for the usual blood constituents (Table I), except for the serum proteins which were slightly low, however. The heavy breeds as barred rocks and the high egg producing white leghorns showed the highest levels of lipids in the blood and tissues.

Some further data obtained from control specimens obtained simultaneously with those of the feeding series are given in the Table II.

⁷ King, E. J., *J. Biol. Chem.* 1932, **26**, 292.

⁸ Phillips, R. A. *et al.*, U. S. Navy Res. Unit at the Hosp. of the Rockefeller Inst. for M. R., 1943.

Experimental Tubular Nephritis Produced by Safranin O

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Following the unexpected death of a rabbit 4 days after the intravenous administration of safranin O,‡ autopsy disclosed somewhat enlarged flabby kidneys with swollen yellowish-streaked cortices§. Microscopic examination of the kidneys revealed severe, essentially selective necrosis of the renal tubular epithelium, involving predominantly the proximal convoluted tubules. The glomeruli appeared uninjured, and there were relatively minor degenerative changes and focal cellular necrobiosis in the loops of Henle and distal convoluted tubules, the collecting tubules appeared normal except for the presence of casts of necrotic material. No noteworthy changes were present in the other organs (brain not examined) except for moderate fatty change and sinusoidal congestion of the liver. The apparent relation of this renal damage to the administration of the dye, and the unexpectedness of this action of safranin, which has been apparently hitherto unreported, prompted a repetition of the procedure for verification and study.

Procedure Healthy stock rabbits weighing about 2 kg were given a single intravenous administration of safranin O|| in physiological saline or in aqueous solution, in a dose of 10, 20 or 40 mg per kg. The rabbits were then sacrificed at intervals for gross and

microscopic study. In most instances determinations of blood NPN were made, these are recorded in Table I.

Observations Immediately following injection of the dye, the tissues took on a distinctly pinkish tinge, this was especially noticeable in the ears, conjunctivae, and mucous and serous membranes. It was readily observed in the skin on parting the fur. The kidneys showed a diffuse staining of the cortex and boundary zone within a minute. The dye appeared in the urine within a few hours and was still present at 24 or even 48 hours, depending on the dose.

From Table I, it may be observed that none of 8 rabbits receiving 10 mg per kg showed any significant change in blood NPN. One animal sacrificed at 24 hours showed mild fatty changes and sinusoidal congestion of the liver, except for some excess of granular debris in the tubular lumens and apparent degenerative changes of somewhat questionable significance in the distal portions of the proximal convoluted tubules, the kidneys were not affected at this time. In 2 sacrificed at 48 hours, the greater portion of the renal parenchyma appeared entirely normal, here and there however, a number of scattered proximal convoluted tubules presented one or several cells showing early but definite coagulation necrobiosis manifested by hyper-eosinophilia of the cytoplasm, and nuclear pyknosis, lysis and karyorrhexis. Many of these cells were in the process of being extruded into the lumen of the tubule, while adjacent cells appeared entirely normal. In all rabbits, at this dose, sacrificed subsequently, no significant lesions were observed, and any pre-existing damage had been repaired without remaining evidence.

At 20 mg per kg, 4 of 8 rabbits showed a distinct rise in blood NPN at the time of sacrifice. In all 4 animals in which this did

* Major, M C, A U S

† With the technical assistance of Elizabeth B Burkemper and Lt Charles Boyers

‡ A basic nuclear stain, one of the azin group of quinone imine dyes, a mixture of dimethyl and trimethyl diminophenylphenazolum chloride

§ The original observation was made by the author in 1942 as an incidental finding in a rabbit submitted for pathological examination by Captain Russel Bowers

|| Safranin O, for general staining purposes. Total dye content 85% C I No 841 National Aniline & Chemical Co, Inc, New York, N Y

TABLE I
Blood NPN of Rabbits Following Intravenous Injection of Safranin O

Dose (mg/kg)	Before injection	NPN (mg/100 cc)								Fate
		1	2	3	4	5	6	7	9	
10	43	46								Sacrificed 1st day
	49		50							2
	51		59							2
	55			44						3
	47					35				5
	39					40				5
	45		48					56	50	9
	47		42					62	50	9
	50	60								1
	39	82								1
20	40		149							2
	49			46						3
	53		55		54					4
	44					181				5
	37					281				5
	44		52				51			Died 5
	45		48				65			Sacrificed 12
	*		138	98						" 4
	—									Died 31 47 hr
	—									" 48 "
40	59		246							Sacrificed 2nd day
	40			258						" 3
	47			157						" 3
	—									Died 72 96 hr
	30				392					Sacrificed 4th day
	—									

* This animal received second injection of 20 mg per kg 12 days after first injection

not occur, examination of the kidneys revealed relatively mild tubular necrosis, limited to the distal segment of the proximal convoluted tubules and involving apparently only a limited number of nephrons. In those in which the NPN rose, there was extension of the necrosis to the proximal as well as the distal portion of the proximal convoluted tubules, and involvement varying in extent from a moderate proportion to almost all of the nephrons (Fig 1). In the most severe, there were also distinct degenerative changes and focal necrobiosis of cells in the ascending limb of Henle and in the distal convoluted tubules as well. In all, there was distinct sparing of the nephrons in the inner half of the cortex as compared with the outer half, I have noted this in experimental mercuric poisoning in rabbits also. Similar moderately severe damage was present in another rabbit which had had a previous injection of this same amount 12 days before, the first injection had resulted in but slight rise in NPN while a more marked elevation followed the second injection (Table I). In a dog

which also received this amount of safranin, the kidneys presented a similar picture of extensive necrosis of the proximal convoluted cortical tubules.

The dose of 40 mg per kg was administered in 2 separate injections within a period of 2-4 hours, as the injection of this amount as a single dose led to slowing and cessation of respiration. With this amount, 3 of 7 rabbits died at 31-47, 48 and 72-96 hours, respectively. All the others showed marked elevation of NPN at the time of sacrifice. The kidneys of all of the animals of this group showed extensive severe tubular necrosis, even as early as 24 hours (not in Table I), the glomeruli remaining unaffected. It was apparent that the severity of renal damage would unquestionably have led to the death of the sacrificed rabbits as well, had they been permitted to continue. In this, as well as in the other groups, there were no noteworthy findings in the other organs, the brain not being examined, except for mild fatty degeneration and sinusoidal congestion of the liver.



Fig 1

Extensive necrosis of epithelium of proximal convoluted tubules in renal cortex of rabbit following intravenous administration of 20 mg per kg of safranin O. On the left a small group of proximal convoluted tubules have escaped damage. The distal tubules are relatively intact except for the presence of casts in the lumen. The glomeruli appear uninjured except for the presence of some granular precipitate in the capsular space of the one at the left.

Discussion The gross observation of the very rapid deposition of safranin in the kidneys has also been reported by Sheehan¹ who likewise administered this substance to rabbits, but failed to observe any renal or other damage, presumably because of the dose he employed which was apparently 5 mg per kg. Sheehan reported that in unfixed frozen sections of the kidney of rabbits sacrificed within one minute after injection of the dye, heavy staining of intermittent nephrons with the dye could be observed whereas the intervening ones might be altogether uncolored. He also showed that at low rates of renal circulation, the extraction ratio of the kidney for the dye is, for practical purposes, 100% and he has concluded that, whether or not there is glomerular filtration of the dye a considerable proportion is secreted by the tubules from the peritubular capillaries.

The histologic picture of the renal injury observed in these animals closely resembles

indeed except perhaps for minor detail, is indistinguishable from that caused by mercuric chloride, chromates, uranium, bismuth, and, under certain conditions, cadmium.² It is of interest, in regard to biochemical mechanisms of tissue injury, that the pathologic-anatomical effects of heavy metal poisoning should be so closely paralleled by an organic compound such as safranin, in addition, essentially the identical histologic picture of renal tubular injury follows the nephrotoxic action of other organic compounds including oxalates and tartrates, and the dyes, the styrol quinoline dye No 90 2(p-acetylamino styryl)6-dimethylamino-quinoline metho-chloride and acriflavine.⁴ The question arises whether similar types of biochemical mechanism of intoxication may not be operative

² Ginzler, A. M., et al., *Proc Am Assoc Path and Bact Am J Path*, in press.

³ Sheehan, H. L., *J Path and Bact*, 1932, **35**, 589.

⁴ Melenev, F. L. and Zin, Z., *J A M A*, 1925, **84**, 337.

¹ Sheehan, H. L., *J Physiol*, 1931, **72**, 201.

Considerable evidence indicates that the toxic action of heavy metals may be due to combination with SH enzymes^{5,6}. That at least some of the organic compounds mentioned above, specifically safranin, acriflavine and the styrol quinoline compound, may likewise act as inhibitors of enzyme systems and in this way exert their damaging action on the kidneys in a manner paralleling the action of the heavy metals biochemically as well as pathologically, is indicated by Dickens⁷ demonstration of a striking and selective inhibition of the normal aerobic inhibition of fermentation by extremely low concentrations of phenosafranin, the chemical structure of which is closely related to that of safranin, and he has demonstrated a like action of acriflavine as well as a quinoline relative of the styrol quinoline compound No 90

Summary The intravenous administration of the dye, safranin O, to rabbits and dogs produces selective renal tubular necrosis, involving predominantly the proximal con-

volute tubules. The histologic picture very closely resembles that observed in heavy metal poisoning.

Safranin O, in a dose of 10 mg per kg, produces minimal histologic injury in the kidney in rabbits, which is readily and rapidly repaired. The intravenous administration of 20 mg per kg is followed by more extensive and severe necrosis of the proximal convoluted cortical tubules, which is, in perhaps half the animals, reflected in elevation of the blood NPN. Increasing the dose to 40 mg per kg results in almost complete necrosis of the renal proximal convoluted tubular epithelium, as well as some damage to the distal tubules, this dose is probably uniformly fatal to rabbits. With all amounts of the substance, noteworthy lesions of other organs than the kidney, in the absence of examination of the brain, appear to be limited to a relatively mild degree of fatty degeneration and sinusoidal congestion of the liver.

The suggestion is made and evidence cited that the nephrotoxic action of safranin, as well as that of certain other nephrotoxic organic compounds, parallels the action of heavy metals biochemically as well as pathologically, and that these organic compounds exert their nephrotoxic effect by acting as inhibitors of vital enzyme systems.

⁵ Peters, R. A., and Thompson, R. H. S., *Nature*, 1945, **156**, 616

⁶ Waters, L. L., and Stock, C., *Science*, 1945, **102**, 601

⁷ Dickens, F., *Biochem. J.*, 1936, **30**, 1233

15285

Potassium Deficiency in the Dog *

W. R. RUEGAMER, C. A. ELVEHJEM, AND E. B. HART

From the Department of Biochemistry, College of Agriculture, University of Wisconsin, Madison

Recent reports by Lambooy and Nasset¹ and Smith^{2,3,4} have indicated that factors, in addition to the better known nutrients, are necessary for optimum growth, prevention

of paralysis, maintenance of a healthy skin and prevention of anemia in the dog. We have found a basal ration containing only the 6 water soluble vitamins (thiamin, riboflavin,

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¹ Lambooy, J. P., and Nasset, E. S., *J. Nutrition*, 1943, **26**, 293

² Smith, S. G., *Science*, 1944, **100**, 389

³ Smith, S. G., *Am. J. Physiol.*, 1944, **142**, 476

⁴ Smith, S. G., *Am. J. Physiol.*, 1945, **144**, 175

TABLE I

	Basal ration (Smith)	Basal ration (modified Strict basal)
Sucrose	36%	66%
Casein	40	19
Cottonseed oil	18	11
Cod liver oil	2	0
Salt mixture	4	4
Thiamin	1.4 mg/dog/day	0.1 mg/kg/day
Riboflavin	0.7 " " "	0.1 " " "
Nicotinic acid	6.0 " " "	2.0 " " "
Calc pantothenate	6.0 " " "	0.5 " " "
Pyridoxine	6.0 " " "	0.06 " " "
Choline chloride	100.0 " " "	50.0 " " "
Inositol	100.0 " " "	0 " " "
p-aminobenzoic acid	6.0 " " "	0 " " "
Vit A	1800 I U " "	1800 I U/dog/day
Vit B	500 " " "	500 " " "
Vit E	15 cc mixed to cephalols/week (Lederle)	0

Smith salt mixture	Salt mixture (Siltz IV—Wis)
Bone meal, steamed	CaCO ₃
Sodium chloride	K ₂ HPO ₄
Lime stone	CaHPO ₄
Iron sulfate	MgSO ₄
Magnesium oxide	NaCl
Copper sulfate	Fe(C ₆ H ₅ O ₇) ₂
Manganese sulfate	KI
Zinc oxide	MnSO ₄
Cobalt carbonate	ZnCl ₂
Potassium iodide	CuSO ₄

nicotinic acid, pyridoxine, pantothenic acid and choline) together with vitamins A and D to be remarkably satisfactory for growing dogs⁵. In fact, excellent growth and blood regeneration have been obtained even when this ration contained levels of succinyl-sulfathiazol as high as 4%⁶. Previous results⁵ have indicated that these differences are not due to variations in the carbohydrate, fat and protein content of the rations nor to the addition of p-aminobenzoic acid and inositol. However, the low choline level in Smith's ration, even in the presence of high protein, might be a limiting factor. In recent work, we⁷ have observed a mortality of 75% in dogs receiving low levels of vitamins in which the choline level was comparable to

that fed by Smith (10 mg/kg body weight/day) and autopsy revealed a fatty infiltration of the liver. When the choline level was raised to 50 mg/kg body weight/day, the animals grew normally and remained in good health.

With this information at hand, an experiment was undertaken in which Smith's basal ration (Table I) was duplicated as nearly as possible and fed to a litter of 6 8-week-old pups. For purposes of comparison the composition of the basal ration used in our previous work is also given in Table I. Two of these animals (dogs 1 and 2) received added amounts of choline (50 mg/kg body weight/day) and one dog (dog 6) was fed 7 µg of biotin/kg body weight/day. All animals were fed ad libitum and received an aqueous suspension of the B vitamins twice a week administered by pipette. Three ml blood samples were obtained from the radial vein on the same day every week before the morning feeding, and the hemoglobin

⁵ Ruegamer, W. R., Michard, L., Elvehjem, C. A., and Hart, E. B., *Am J Physiol*, 1945, 145, 23.

⁶ Michard, L., Mars, A. R., Ruegamer, W. R., and Elvehjem, C. A., *Proc Soc Exp Biol and Med* 1944, 56, 148.

⁷ Unpublished data.

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Sucrose	36%	66%
Cocain	40	19
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Cod liver oil	2	0
Salt mixture	=	4
Thiamin	1 1/2 mg/dog day	0.1 mg/kg/day
Riboflavin	0.7	0.1
Nicotinic acid	6.0	2.6
Calc pantothenic	6.0	0.5
Pyridoxine	6.0	0.00
Choline chloride	100.0	50.0
Inositol	100.0	0
p-aminobenzoic acid	0.0	0
Vit. A	1,800 I.U.	1,800 I.U. dog day
Vit. B	500	500
Vit. E	5 cc mixture of (spherulic ester) (Lederle)	0

Smith salt mixture		Salt mixture (Salts IV—Wisc)	
Bone meal steamed	57.8%	CaCO ₃	32.3%
Sodium chloride	24 1/2	K ₂ HPO ₄	34.75
Lime stone	12.2	CaHPO ₄	5.35
Iron sulfate	3.7	MgSO ₄	0.67
Magnesium oxide	1.2	NaCl	18.04
Copper sulfate	0.3	Fe(C ₆ H ₅ O-) ₂	2.37
Manganese sulfate	0.1	KI	0.08
Zinc oxide	0.1	MnSO ₄	0.26
Cobalt carbonate	0.1	ZnCl ₂	0.03
Potassium iodide	0.1	CaSO ₄	0.02

nicotinic acid pyridoxine pantothenic acid and choline) together with vitamins A and D to be remarkably satisfactory for growing dogs.⁵ In fact excellent growth and blood regeneration have been obtained even when this ration contained levels of succinyl-sulfathiazol as high as 4%.⁶ Previous results⁵ have indicated that these differences are not due to variations in the carbohydrate and protein content of the rations nor to the addition of p-aminobenzoic acid and inositol. However the low choline level in Smith's ration even in the presence of high protein might be a limiting factor. In recent work we⁷ have observed a mortality of 75% in dogs receiving low levels of vitamins in which the choline level was comparable to

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⁷ Unpublished data.

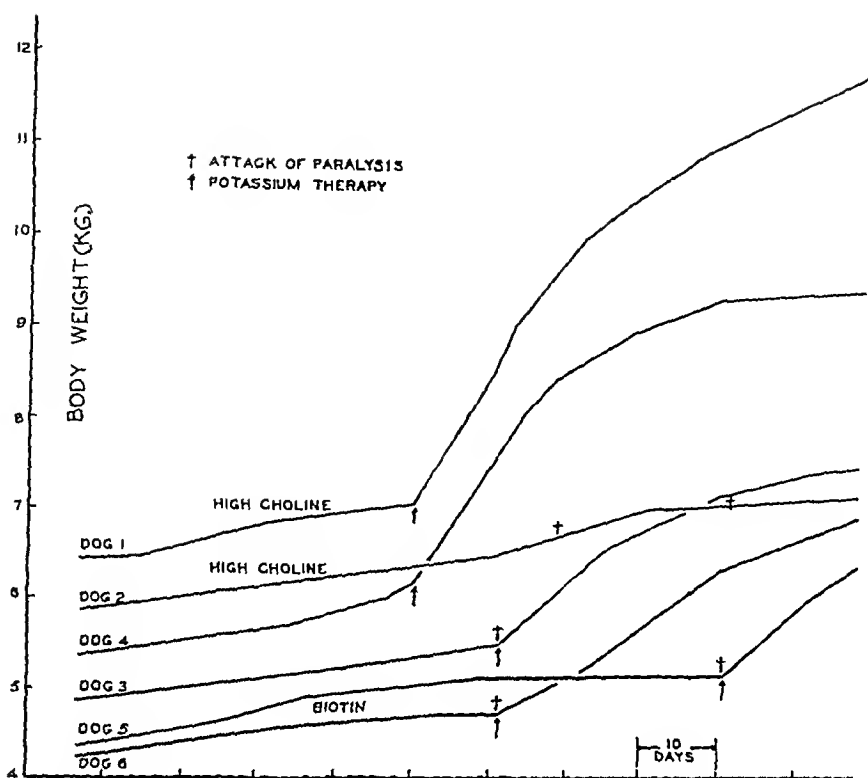


Fig 1
The growth response of dogs on the Smith basal ration with and without added potassium

level determined by the method previously used^a

Results All animals showed poor growth and a plateau in the growth curves was soon evident (Fig 1). After a few weeks the animals became restless and began to develop symptoms very similar to those described by Smith. At this time, the ration was checked and found to be very low in potassium. In spite of the fact that Smith mentions the administration of potassium chloride to one animal in 100 mg doses every hour for 7 hours without apparent effect, it was decided to try potassium in the case of two of the animals. A single dose of potassium chloride (3 g) was given by capsule to dogs 1 and 4, and the animals continued on the Smith ration but the salt mixture was changed to contain 160 g of KCl/1000 g of salt mixture. An immediate growth response occurred which continued for 30-40 days. One of the animals (dog 1), which received the potas-

sium supplement had been on the high choline level from the start of the experiment and had exhibited no growth until potassium therapy was started.

The remainder of the animals were kept on the Smith basal ration until dog 6, receiving supplements of crystalline biotin, developed a moderate attack of paralysis. The paralysis first started in the muscles of the neck and the animal developed what might be termed a "stiff neck" and was unable to hold its head erect. Several hours later, the animal was given 3 g of potassium chloride by capsule and was placed on the potassium supplemented ration, as in the case of dogs 1 and 4. This animal likewise showed a very marked growth response of approximately 200 g per day, and the paralytic symptoms disappeared completely.

Two days later, dog 3 developed a neck paralysis which grew progressively worse until the animal became severely paralyzed.

about 10 hours later. The rear legs were completely paralyzed and the front legs became so weak that the dog was unable to raise itself from the bottom of the cage. At this point 3 g of potassium chloride was given by capsule and one hour later the animal was able to stand and walk around. This animal was also placed on the potassium supplemented Smith ration and failed to develop any recurrent attacks of paralysis.

Dog 2 (high choline ration) showed 2 attacks of paralysis which were relatively mild and involved only the neck region. The first attack suffered by dog 5 (low choline ration) involved only the neck, but the second attack was quite severe. Three g of potassium chloride was given by capsule and the dog was placed on the potassium supplemented ration. This animal responded well to the potassium therapy and experienced no further transient attacks.

Hemoglobin values ranged between 15 and 16 g per 100 cc of blood just before the animals developed paralysis. Since these animals were only $3\frac{1}{2}$ -4 months old at this time, it is possible that a hemoconcentration existed as a result of a salt imbalance. After the administration of potassium, the hemoglobin levels dropped to 13-14 g per 100 cc.

As the experiment progressed and before potassium supplements were given, the teeth of all dogs became discolored and the enamel appeared to be eroded. With potassium therapy this condition appeared to improve somewhat but recovery was not complete.

Discussion When Smith's ration was duplicated as nearly as possible and fed to litter-mate dogs, all animals ceased to grow and eventually developed paralysis. When potassium therapy was started all dogs demonstrated a rapid growth response of 200-250 g per day and the paralysis disappeared.

It is interesting to note that choline appears to have no effect on the deficiency syndrome observed, since added amounts of choline failed to stimulate growth and prevent the onset of paralysis. It should be pointed out that even though dog 6 received 7 μ g of crystalline biotin per kg body weight per day throughout the experiment, this animal suffered attacks of paralysis and failed to

grow until potassium therapy was started.

From these data, it would appear improbable that a multiple deficiency developed since the administration of potassium alone brought about an immediate growth response of 200-250 g per day. If there had been a multiple deficiency it would seem that there should have been an initial response to potassium and that further deficiency would have prevented the animals from making such dramatic weight gains over such an extended period.

The cardiac failure observed so frequently by Smith is another point in favor of an existing potassium deficiency since cardiovascular lesions have frequently been found in rats fed diets deficient in potassium⁸.

In the light of this work, it is difficult to explain the results obtained by Smith with biotin. However in all cases, the biotin seemed to have only a temporary beneficial effect and the animals soon relapsed into a state of paralysis. Only when the animals were placed on a ration containing 10% yeast which would contain ample amounts of potassium, did the animals recover completely from the paralysis and return to a normal state of health. It is possible, though unlikely, that the administration of potassium increases the intestinal synthesis of biotin, which is actually the curative agent and that our biotin dog did not receive sufficient amounts of crystalline biotin to prevent paralysis.

If the deficiency syndrome observed is due to a potassium deficiency, the paralysis might be explained on the basis of a breakdown in certain enzyme systems. Nachmansohn *et al*⁹ have suggested that potassium seems to be concerned with *in vivo* enzyme systems responsible for phosphorylation. This theory was further substantiated by the work of Boyer *et al*¹⁰ who found that potassium markedly accelerates the transfer of phosphate from 3-phosphoglycerate to creatine in

⁸ McCollum, E. J., Orent Keles, E., and Day, H. G., *The New Knowledge of Nutrition* 5th ed., The Macmillan Company, New York, 1939, 203.

⁹ Nachmansohn, D. and John, H. M., *Proc. Soc. Exp. Biol. and Med.*, 1944, **57**, 361.

¹⁰ Boyer, P. D., Lardy, H. A., and Phillips, P. H., *J. Biol. Chem.* 1943, **149**, 529.

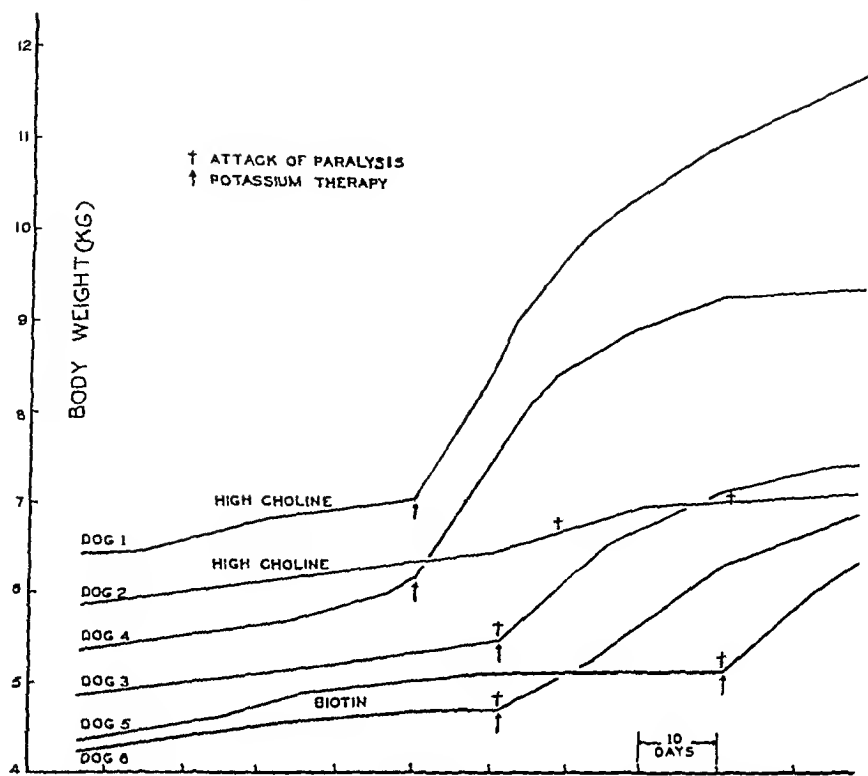


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Two days later, dog 3 developed a neck paralysis which grew progressively worse until the animal became severely paralyzed.

S. dispar, TYPES I, II AND *S. paradysenteriae*, BOYD TYPE P143

TABLE I
Cross agglutination and Reciprocal Adsorption of *Sh. dispar*, Types I and II, and
Sh. paradysenteriae, Type P143

Serum	Adsorbed with strain	Agglutinative titer with				
		<i>Sh. dispar</i> serotype				<i>Sh. para</i> P143
		I (171)	II _a (167)	II _b (205)	II _c (231)	
171	Unads	5120*	640	1280	1280	80
	167	5120	0†	0	0	80
	205	5120	0	0	0	80
	231	5120	0	0	0	40
	P143	2560	640	640	1280	0
167	Unads	640	81920	40960	40960	640
	171	0	20480	5120	10240	160
	205	0	2560	0	2560	320
	231	0	0	0	0	0
	P143	320	20480	20480	20480	0
205	Unads	320	10240	10240	10240	20
	171	0	10240	5120	10240	20
	167	0	0	0	0	0
	231	0	0	0	0	0
	P143	160	10240	5120	5120	0
231	Unads	1280	10240	10240	10240	320
	171	0	10240	10240	5120	160
	167	0	0	0	160	0
	205	0	80	0	2560	40
	P143	1280	10240	10240	10240	0
P143	Unads	1280	2560	2560	2560	10240
	171	0	320	1280	640	2560
	167	320	0	0	0	2560
	205	80	160	0	20	2560
	231	160	0	0	0	2560

* Titers are expressed as reciprocals of the highest dilutions giving any agglutination after 15-18 hours incubation at 55°C

† 0 indicates no agglutination in 1:40 dilution

TABLE II
Antigens of *Sh. dispar* Types I and II, and *Sh. paradysenteriae*, Type P143

Culture No	Type	Antigens
171	<i>Sh. dispar</i> I	A C
167	<i>Sh. dispar</i> II _a	AB D
205	<i>Sh. dispar</i> II _b	AB
231	<i>Sh. dispar</i> II _c	AB DE
P143	<i>Sh. paradysenteriae</i> P143	BCD F
2-193		AB

alkalescens Neter^{3,4} has recently so classified this strain. However, he stated that it does not share any major antigens with *Sh. alkalescens* types I, II or IV, and established a separate serotype (III) for it. He noted that this strain is strongly agglutinated by antiserum P143. Wheeler *et al.*⁷ reported strong cross reactions between culture 2-193

(Wheeler's No 2372) and type P143 and postulated a common antigen distinct from the specific P143 antigen. They also noted very close relationship between 2-193 and *Sh. dispar*, type II.

In the present investigation, strain 2-193 agglutinated in 1:2560 dilution of antiserum P143 (homologous titer 1:10,240). Conversely, P143 agglutinated to a titer of 1:320 in an antiserum for 2-193 (supplied by Dr. Wheeler) whose homologous titer was 1:20,480. By use of monovalent serums prepared by suitable adsorption (Table II), major antigens A and B were found in strain 2-193. It is therefore antigenically a typical *Sh. dispar*, type II_b. It is to be noted that fraction B is the antigen common to 2-193 and P143. Furthermore, culture 2-193 agglutinated to titer in antiserum 205 (type II_b) and almost completely adsorbed the latter (titer reduced from 20,480 to 160).

⁷ Wheeler, K. M., Stuart, C. A., and Ewing, W. H., *J. Bact.*, 1946, in press.

homogenized fresh muscle, and that potassium is necessary for the phosphorylation of creatine to accompany pyruvate oxidation by animal tissues. Thus the paralysis may be due to a breakdown in the enzyme system responsible for muscular contraction.

Potassium therapy has been of some value in clinical investigations of certain types of paralysis. Aycock¹¹ has recently reported a case of familial paralysis in which the administration of potassium brought about a prompt recovery from the paralysis. Likewise, potassium chloride has been found to have a mild effect on the symptoms of

myasthenia gravis. Several dog breeders and veterinarians have called our attention to the frequent existence of a paralysis in animals maintained on commercial dog foods. The possibility of a potassium deficiency existing in these rations is being studied at the present time.

Summary In order to study the paralysis described in dogs by Smith, a ration similar to that described in her work was prepared and fed to growing dogs. When placed on this ration, our animals failed to grow and developed a paralysis which was curable with potassium. Even though Smith has obtained cures with the administration of biotin, one of our animals receiving biotin became paralyzed and responded to potassium therapy.

¹¹ Aycock, W. L., and Foley, G. E. *Am J Med Sc*, 1945, **210**, 397.

15286 P

Antigenic Relationship of *Shigella dispar*, Types I and II, to *Shigella paradysenteriae*, Boyd Type P143

PHILIP L. CARPENTER AND C. A. STUART

From the Department of Bacteriology, Rhode Island State College, Kingston,
and the Biological Laboratories, Brown University, Providence

Attempts to classify certain paradysentery-like bacteria have indicated a possible antigenic relationship between *Shigella dispar* and *Shigella paradysenteriae*, type P143.¹ In particular, a culture (No. 2-193) isolated by Major William H. Ewing, Sanitary Corps, United States Army, has been classified on various grounds as *Sh. paradysenteriae*, type P143,² *Sh. alkalescens*,^{3,4} and *Sh. dispar*.² The present investigation was undertaken to attempt to clarify the relationship between *Sh. dispar* and type P143.

Four serotypes and several subtypes of *Sh. dispar* were previously reported.⁵ Representative strains of these were employed. Type P143 was secured through the courtesy

of Dr. K. M. Wheeler of the Connecticut Public Health Laboratories as his culture No. 573.⁶ Antiserums were prepared by immunizing rabbits with living vaccines of this and of the type and subtype strains of *Sh. dispar*.

Cross agglutination tests indicated the existence of antigens common to type P143 and *Sh. dispar*, types I and II. This was confirmed by reciprocal adsorption of the cross-reacting serums, as shown in Table I. From these data were derived the partial antigenic formulae listed in Table II. The relationship between *Sh. dispar*, types I and II, and *Sh. paradysenteriae*, type P143, is apparent. While antigens B, C and D are apparently minor factors in P143, nevertheless, they appear to account for the strong agglutination (to titers of 1280 or 2560) of types I and II in antiserum P143.

Culture 2-193 is biochemically like *Sh.*

¹ Boyd, J. S. K., *J. Hyg.*, 1938, **38**, 477.

² Ewing, W. H., personal communication.

³ Neter, E., *Proc. Soc. Exp. Biol. and Med.*, 1944, **57**, 200.

⁴ Neter, E., *J. Imm.*, 1945, **51**, 151.

⁵ Carpenter, P. L., *J. Bact.*, 1944, **47**, 419.

⁶ Wheeler, K. M., *J. Imm.*, 1944, **48**, 87.

tests^{12,13} These observations support the previously advanced hypothesis that PABA inhibits the multiplication of rickettsiae inside the cells, thereby permitting the immunity mechanisms of the body to dispose of them¹² The treatment of these rickettsial infections with PABA, therefore, should not interfere with the development of immunity to them This has been shown to be true in guinea pig experiments with Rocky Mountain spotted fever¹³ In order to obtain additional data, however, immunity tests were carried out in gerbilles which had survived inoculation with scrub typhus with the aid of PABA therapy The purpose of this report is to give the results of these tests

Immunity Tests Six to 9 months after the PABA therapy experiments were concluded there were 69 treated gerbilles available for immunity tests Of these, 24 were from the Ceylon strain experiments, 4 from the Calcutta, 32 from the Karp and 9 from the Imphal strain experiment The gerbilles had gained weight and appeared healthy at the time of testing for immunity to their respective homologous strains

The test inocula consisted of peritoneal washings from one or more gerbilles which had been infected with the strain desired Three cubic centimeters of sterile physiological saline solution were used to make the peritoneal washings, and, from this, serial ten-fold dilutions were made Each gerbille was inoculated intraperitoneally with 1 cc of the 10^{-2} dilution of homologous strain peritoneal washings Simultaneously, each strain inoculum was titrated in normal gerbilles in dilutions from 10^{-1} through 10^{-6} The animals were then observed for 21 days, at which time the experiment was arbitrarily closed

There was but one death in the 69 gerbilles tested, though the animals had received from 2,140 to over 3,600 lethal doses of infectious material The single death occurred in a gerbille of the Karp strain group It died on the 11th post-inoculation day and autopsy was not at all suggestive of scrub typhus The liver and spleen appeared normal and there was no pleural effusion Large numbers of bacteria were seen in smears of

the peritoneal exudate

It appears, then, that these gerbilles which had been infected with scrub typhus and survived through treatment with PABA developed a strong immunity to the homologous strain of *R. orientalis* It should be noted, however, that Fox and Peterson have found that *R. orientalis* persist up to a year or more in mice which survived either as a result of methylene blue treatment or as a result of infection by subcutaneous inoculation¹⁴ The presence of active organisms in the cells of an infected animal might well give rise to 'interference phenomenon' so that subsequent survival to re-inoculation might not represent immunity in the usual sense of the term The presence of surviving rickettsiae from primary inoculation was not investigated in the gerbilles used in these tests However, the fact that the gerbilles resisted challenge indicates that infection was established on primary inoculation in spite of PABA treatment If immunity developed in these gerbilles and is the reason for their survival, then, the effect of PABA was to inhibit the rickettsiae permitting the mechanisms of immunity to develop Should living *R. orientalis* be demonstrated in animals surviving infection with the aid of PABA treatment as it has been in the case of methylene blue treated mice, the conclusion would again be that the effect of PABA was rickettsiostatic rather than rickettsiocidal

Cross-immunity Tests Cross-immunity between various strains of *R. orientalis* has been demonstrated on several occasions The literature on this subject has been recently reviewed by Blake, *et al*¹⁵ who also investigated cross-immunity with other strains Additional strains have since been tested by other workers¹⁶ However, cross-immunity has not been described for the 4 strains involved in these PABA studies Therefore, upon the completion of the immunity tests

¹⁴ Fox, J and Peterson, O, personal communication

¹⁵ Blake, F G, Mavey, K F, Sadusk J F, Jr, Kohls, G M, and Bell, E J, *Am J Hyg*, 1945, **41**, 243

¹⁶ Kohls, G M, Armbrust, C A, Irons, E N, and Philip, C B, *Am J Hyg*, 1945, **41**, 374

Likewise, antiserum 2-193 agglutinated strain 205 to titer and when adsorbed with 205 had a residual homologous titer of only 40

It thus appears that antiserum P143 may

be rendered more nearly monovalent by adsorption with *Sh dispar*, type I and IIa or IIc This procedure would prevent confusion in classification of cultures such as 2-193

15287

Immunity Following Para-Aminobenzoic Acid Therapy in Experimental Tsutsugamushi Disease (Scrub Typhus)

C J D ZARAFONETIS, J C SNYDER, AND E S MURRAY
(Introduced by S Bayne-Jones)

From the Cairo Unit of the United States of America Typhus Commission

Two recent communications^{1,2} from this laboratory have dealt with the therapeutic effectiveness of para-aminobenzoic acid (PABA) in the treatment of experimental tsutsugamushi disease The beneficial effect of PABA and especially that of the sodium salt of the acid (NaPAB) was demonstrated against the Ceylon, Calcutta, and Karp strains of *R orientalis*^{1,2} and later against an Imphal strain³ The animals used in these experiments were 2 species of desert rodents, *Gerbillus pyramidum* and *Gerbillus gerbillus*, which are abundant in Egypt⁴ The susceptibility of gerbilles to these strains of *R orientalis* and the manifestations of the disease in them have been detailed elsewhere⁵

Para-aminobenzoic acid has also been shown experimentally to have a beneficial therapeutic effect on murine⁶⁻⁹ and epidemic typhus,⁷

and on Rocky Mountain spotted fever^{10,11} Clinical trial of PABA has demonstrated it to be of value in the treatment of louse-borne typhus fever¹² There has also been recently reported a case of Rocky Mountain spotted fever which received PABA with apparent benefit¹³

Thus, it has been shown by several investigators that PABA has an effect on several rickettsial agents The mode of action of PABA on these organisms is of great interest since they are obligate intracellular parasites Various studies carried out in developing chick embryos indicate that PABA is rickettsiostatic rather than rickettsiocidal⁷⁻¹⁰ Furthermore, in PABA treated human cases of epidemic typhus fever and the case of Rocky Mountain spotted fever there was no apparent disturbance of the antibody dynamics as judged from findings in Weil-Felix and complement-fixation

¹ Snyder, J C, and Zarafonetis, C J D, *Proc Soc Exp Biol and Med*, 1945, **60**, 115

² Murray, E S, Zarafonetis, C J D, and Snyder, J C, *Proc Soc Exp Biol and Med*, 1945, **60**, 80

³ Unpublished observations of the authors

⁴ Snyder, J C, Zarafonetis, C J D, and Liu, W T, *Proc Soc Exp Biol and Med*, 1945, **59**, 110

⁵ Zarafonetis, C J D, *Proc Soc Exp Biol and Med*, 1945, **59**, 113

⁶ Snyder, J C, Muir, J, and Anderson, C R, Report to the Division of Medical Sciences, National Research Council, Washington, D C, December 26, 1942

⁷ Hamilton, H L, Plotz, H, and Swedel, J E,

Proc Soc Exp Biol and Med, 1945, **58**, 255

⁸ Greiff, D, Pinkerton, H, and Moragues, V, *J Exp Med*, 1944, **80**, 561

⁹ Greiff, D, and Pinkerton, H, *J Exp Med*, 1945, **82**, 193

¹⁰ Hamilton, H L, *Proc Soc Exp Biol and Med*, 1945, **59**, 220

¹¹ Anigstein, L, and Bader, M N, *Science*, 1945, **101**, 591

¹² Yeomans, A, Snyder, J C, Murray, E S, Zarafonetis, C J D, Ecker, R S, *J A M A*, 1944, **126**, 349

¹³ Rose, H M, Durine, R B, and Fischel, D E, *J A M A*, 1945, **129**, 1160

or artificial forms. Whether these are minor components of the flagellar complex brought into prominence or artifacts produced by action of the serum has been the subject of some discussion. Hitherto, such forms have not been recognized in nature, although Eriksson and Malmstrom⁷ found agglutinins for an induced phase of *S. newport* in the serum of a patient infected with that type. The present note records the occurrence in nature of antigens apparently identical with those obtained by cultivation of *Salmonella* in agglutinating serum.

Since April, 1945, 14 cultures received from Connecticut, Maryland, Florida, Illinois, California and Uruguay, were apparently monophasic variants of *S. minnesota*, which had the formula XXI, XXVI b Phase 2 (e,n,\) could not be demonstrated. When stabbed into semi-solid agar containing b serum, the cultures were either confined to the site of inoculation or produced one or 2 small bulbs of spreading growth which yielded a form flocculated no longer by serums for the known H antigens of the genus but by serums for certain induced antigens. By continued transfer in b serum, such forms were obtained from 13 of the cultures. While this work was in progress, 2 cultures isolated from sewage by Mr. A. A. Hajna were received. These cultures also had O antigens XXI, XXVI and gave H reactions similar to those cited above. Serums were prepared from one of the induced forms of the XXI, XXVI b cultures and one of the sewage cultures. Reciprocal agglutination and absorption tests indicated that the forms obtained by growth in b serum were identical with the cultures from sewage. A symbol (z_{33}) was assigned the H antigens of these forms. By cultivation of the z_{33} form of the XXI, XXVI b cultures in z_{33} serum, e,n,\ phases were obtained from 5 of them. One reverted to b. The 2 sewage cultures (XXI, XXVI z_{33}) were cultivated in z_{33} serum and an e,n,\ phase was obtained from one. The z_{33} and e,n,\ forms are quite resistant to change. As yet a change from b directly to e,n,\ has not been accomplished. The

changes brought about in the 2 naturally occurring forms may be summarized as follows:

XXI, XXVI b \rightarrow XXI, XXVI z_{33} \rightarrow XXI, XXVI e,n,\

XXI, XXVI z_{33} \rightarrow XXI, XXVI e,n,\

Since so little is known concerning phylogeny and evolution in the *Salmonella*, it is difficult to interpret these results. A complex parent containing b, e,n,\ and z_{33} could be postulated for *S. minnesota* (XXI, XXVI b-e,n,\) and the XXI, XXVI b and XXI, XXVI z_{33} forms. On the contrary it is possible that the XXI, XXVI b cultures are loss variants of *S. minnesota* which give rise to XXI, XXVI z_{33} forms under certain environments. The fact that in the progression b \rightarrow z_{33} \rightarrow e,n,\ only z_{33} gave rise to the e,n,\ phase which would normally be expected in these cultures, indicates that z_{33} is not an artifact or denatured antigen, but a minor constituent brought into prominence. Other antigens hitherto observed only in cultures exposed to agglutinating serum probably also occur in nature. These observations agree with those of Monteverde and Leiguarda⁸ and of Edwards⁹ that *S. ballerup* (XXIX[V] z_{14}) can be changed to a form resembling *S. hormaechei* (XXIX[V] $z_{30,z_{31}}$). They are further supported by unpublished results of the writers, who succeeded in changing all of 9 cultures of *S. simsbury* (I, III, XIX z_{27}) to forms serologically indistinguishable from *S. senftenberg* (I, III, XIX g,s,t). It is not yet possible to say whether the transformability of these apparently distinct types means that serologically divergent strains of *Salmonella* may arise by processes analogous to induced variation or whether these apparently distinct types are merely loss variants of complex parents. In the transformation of *S. salmatis* (IV, XII d,e,h-d,e,n, z_{15}) to *S. sandiego* (IV, XII e,h-e,n, z_{15})⁶ and in the appearance of XXIX[V] z_{30} and XXIX[V] z_{31} variants of *S. hormaechei*,⁹ only loss variation seems operative. In any event, a re-examination of some of the apparently distinct *Salmonella* types is indicated.

⁸ Monteverde, J. J., and Leiguarda, R. H., *Nature*, 1944, 153, 589.

⁹ Edwards, P. R., *J. Bact.*, in press.

⁷ Eriksson, E., and Malmstrom, F., *Z. Hyg.*, 1939, 121, 629.

of the PABA treated gerbilles with their respective homologous strains of *R. orientalis*, cross-immunity tests were performed with heterologous strains. Inocula were prepared and administered in the same manner as described for the immunity tests. Simultaneous titrations of the infectious material were made in normal gerbilles with dilutions of 10^{-1} through 10^{-8} . The test period of 21 days remained the same.

Of the 24 gerbilles immune to the Ceylon strain, 9 were tested with the Karp strain, 8 with the Calcutta, and 7 with the Imphal strain. One death occurred in the group challenged with the Calcutta strain, 15 days after the inoculation. This gerbille showed evidence of marked weight loss, but the autopsy was otherwise negative. No rickettsiae were seen in peritoneal smears.

Three gerbilles immune to the Calcutta strain were challenged with Karp strain inoculum and survived.

The 30 Karp-immune gerbilles were divided into 3 groups. 9 were inoculated with the Ceylon strain, 10 with the Calcutta strain, and 11 with Imphal strain infectious material. There were no deaths among these gerbilles.

The 9 gerbilles immune to the Imphal strain were divided into 3 groups of 3 which

were tested with the Ceylon, Calcutta and Karp strains, respectively. All of the gerbilles survived.

The gerbilles which were tested with the Karp strain received 316,000 lethal doses, those tested with the Calcutta strain received 31,600 lethal doses, while those receiving Ceylon and Imphal infectious material survived 2,140 and 3,160 lethal doses, respectively. Thus, it is apparent that a solid immunity against heterologous strains of *R. orientalis* was present in these gerbilles. It is possible, of course, "interference phenomenon" may have exerted an effect of undetermined degree in these tests.

Summary Gerbilles which were infected with tsutsugamushi disease and survived through treatment with PABA were tested 6 to 9 months later and found to be immune to homologous strains of *R. orientalis*. This is interpreted as evidence that the mode of action of para-aminobenzoic acid in scrub typhus is rickettsiostatic.

Cross-immunity tests revealed that these gerbilles were also immune to heterologous strains of *R. orientalis*.

The technical assistance of Cpl George Trisher and Sgt Robert Stearns is gratefully acknowledged.

15288 P

Natural Occurrence of an "Induced" Antigen in Salmonella Cultures *

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It is known¹⁻⁶ that H antigens of *Salmonella* can be markedly changed by cultivating the organisms in serum containing agglutinins for the flagellar components. Excepting *S. abortus-equi*⁴ and *S. paratyphi A*,⁵ all antigens

so obtained have had little or no relationship to naturally occurring H antigens of the genus, and have been referred to as induced

³ Kruffmann, F, and Tesdal, M, *J. Hyg.*, 1937, 120, 168

⁴ Edwards, P R, and Bruner, D W, *J. Bact.*, 1939, 38, 63

⁵ Bruner, D W, and Edwards, P R, *J. Bact.*, 1941, 42, 467

⁶ Edwards, P R, and Bruner, D W, *J. Bact.*, 1942, 44, 289

* The investigation reported in this paper is in connection with a project of the Kentucky Agricultural Experiment Station and is published by permission of the Director.

¹ Kauffmann, F, *Z. Hyg.*, 1936, 119, 104

² Gnosspecht, A, *Z. Hyg.*, 1939, 121, 529

TABLE II
Production of Staphylococcal Enterotoxin and Alpha Hemolysin in Chemically Defined Media

		Staphylococcal enterotoxin						
		Monkey feeding test			Intravenous cat test			Alpha hemolysin M H D per ml
Basic*	Modification	Dosage (ml per monkey)	Reaction pos	Reaction neg	Dosage (ml per kg body wt)	Reaction pos	Reaction neg	
No 1		50	0	5	10 20	10	3	4 16
	+ proline M/690	50	0	4	10	2	2	64 128
	+ " M/280	50	6	3	10	6	3	32 64
	+ " M/280							
	— aspartic acid							
	glutamic "	50	1	0				4 16
	histidine							
No 2		40 100	2	2	20	1	2	32
	+ proline M/690	50	0	2	20	2	2	16 32
	+ glutamic acid							
	M/280	40 100	1	2	20	2	1	64
	+ hydroxyproline							
	M/280	40 100	1	3	20	2	1	16 32
No 3		55 100	0	3	20	0	4	<4
	+ (NH ₄) ₂ SO ₄ M/250	50 100	0	3	20	1	3	4 16
	+ glycine M/198	40 100	0	3	20 30	3	1	8

* Numbers correspond to Table I

were read after incubation for one hour at 37°C followed by one hour in an ice water bath

Results of tests for enterotoxin and alpha-lysin are shown in Table II. Alpha hemolysin production and vomiting reactions in cats were obtained with cultures grown in all media except Medium No 3. In the latter medium negative results may be correlated with nitrogen depletion, since addition of (NH₄)₂SO₄ or glycine increased growth hemolysin titers and enterotoxic reactions. Four cats failed to react to centrifugates of the nonenterotoxic strain grown in Medium No 1. Monkeys reacted irregularly to centrifugates of cultures grown in the more

complex media. The positive tests are significant since they demonstrate toxicity following feeding. Beta-lysin was detected in centrifugates of beta-positive strains in titers ranging from 8 to 256 M H D (for sheep rbc) per ml.

In general growth and hemolysin production decreased with simplification of the media. Qualitative tests suggest a similar decrease in enterotoxin production.

Summary Production of staphylococcal enterotoxin in chemically defined media is reported. These experiments have not demonstrated specific requirements for enterotoxin production.

Production of Staphylococcal Enterotoxin in Chemically Defined Media *

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Production of staphylococcal enterotoxin in synthetic media has not been previously reported, although Favorite and Hammon¹ and Hammon² used successfully a casein hydrolysate medium. The present is a report of enterotoxin production in synthetic media modified from those used by Gladstone³ in studies of the hemolysins.

Four enterotoxigenic strains (Nos 8, 147, 161 and 196) and one non-enterotoxigenic strain (No 184) of staphylococci were used in these experiments. All strains are alpha-hemolytic and strains No 184 and 196 produce potent beta lysin.

The compositions of 3 basic media used in these experiments are given in Table I. Media were adjusted to pH 7.6, dispensed into nursing bottles or flasks and sterilized in the

autoclave. Inoculations were made with 0.1 ml of a 1:1000 dilution (in phosphate buffer) of culture in synthetic medium.

Cultures were grown at 37°C in an atmosphere of 20% CO₂. During incubation they were rotated continuously on a vertical wheel. Centrifugates of 3 to 7 day cultures were tested for enterotoxin and hemolysins.

Enterotoxin was assayed by the monkey (*Macacca mulatta*) feeding and intravenous cat tests.^{4,5} Untreated centrifugates were fed to monkeys, preparations heated for 30-35 minutes in a boiling water bath were injected into cats. Vomiting was used to indicate a positive reaction. Alpha-hemolysin titers are expressed in terms of the MHD (minimal hemolytic dose) of toxin.⁶ Beta-lysin tests were made against sheep erythrocytes and

TABLE I
Composition of Basic Media

Amino acids	Concentration	Medium 1	Medium 2	Medium 3
l (+) arginine HCl	M/110	+	+	+
l cystine	M/10,000	+	+	+
dl alanine	M/1500	+	+	
glycine	M/198	+	+	
dl phenylalanine	M/4000	+	+	
dl valine	M/1500	+	+	
l aspartic acid	M/1500	+		
l glutamic acid	M/1500	+		
l histidine 2HCl	M/4000	+		
l hydroxyproline	M/1500	+		
l leucine	M/1500	+		
l lysine 2HCl	M/4000	+		
dl methionine	M/4000	+		
l tryptophane	M/20,000	+		
l tyrosine	M/4000	+		

The media also contained M/6000 MgSO₄, M/20,000 FeSO₄ (NH₄)₂SO₄ 6H₂O, M/30 KH₂PO₄, M/37 NaOH, M/80 glucose, 123 µg/ml nicotinic acid, 0.0337 µg/ml thiamine HCl, 10 µg/ml calcium pantothenate (omitted from Media No 1 and No 2 in early tests).

* This work was supported by a grant from the National Cannery Association.

¹ Favorite, G O, and Hammon, W McD, *J Bact*, 1941, **41**, 305.

² Hammon, W McD, *Am J Public Health*, 1941, **31**, 1191.

³ Gladstone, G P, *Brit J Exp Path*, 1938,

19, 208.

⁴ Davison, E, Dack, G M, and Cary, W E, *J Infect Dis*, 1938, **62**, 219.

⁵ Dack, G M, 1943, *Food Poisoning*, University of Chicago Press, Chicago.

⁶ Dolman, C E, and Kitching, J S, *J Path and Bact*, 1935, **41**, 137.

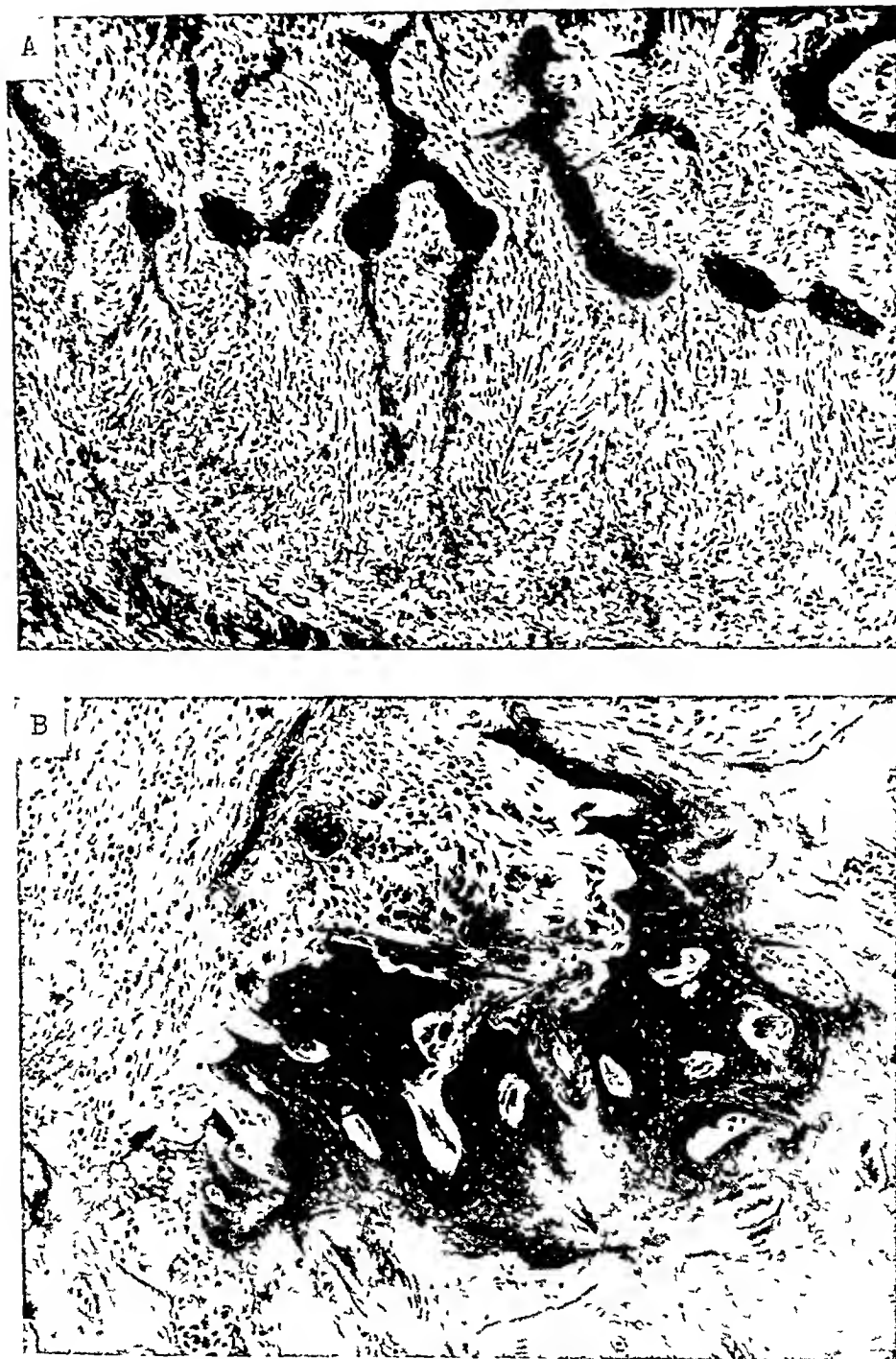


Fig 1

a	Chicken N 3447	Bone tumor	Hematoxylin Eosin	Mag	X 145
b	Chicken N 3449	Bone tumor	Hematoxylin Eosin	Mag	X 135

Bone Tumors in Fowls Injected Intravenously with Causative Agent of Rous Sarcoma

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It is well known that intravenous injections of the causative agent of filterable fowl sarcoma into fowls never produce widespread sarcomatosis and, in the absence of a primary tumor, seldom cause solitary sarcomas in the visceral organs.¹⁻⁷ The agent introduced into the blood stream rapidly disappears from the circulation, probably being taken up by the cells of the reticulo-endothelial system.⁸ There is general belief that the sarcoma producing agent injected into the circulation initiates a new growth only at points of vascular injury, *i.e.* where the agent comes into contact with the connective tissue cells. This can take place in the ovary as a sequence of frequent injuries to the vessels occurring in this organ during the process of ovulation.¹ Experimentally the agent can be localized by thermo-cauterisation of the skin,³ by injections of histamine,⁶ by puncture of the vessel,² by inducing the development of reaction tissue,⁴ etc.

We wish to report in the following our experiments on intravenous injections of the causative agent of Rous sarcoma into chicks, the results of which deviate considerably from previous observations.

In the present study we used White Leghorn chicks of both sexes from the same farm.

¹ Rous, P., Murphy, J. B., and Tytler, W. H., *J. A. M. A.*, 1912, **58**, 1751.

² Pentimalli, F., *Z. f. Krebsforsch.*, 1924, **22**, 74.

³ Findlay, G. M., *J. Path. and Bact.*, 1928, **31**, 633.

⁴ Mackenzie, R. D., and Sturm, E., *J. Exp. Med.*, 1928, **47**, 345.

⁵ Cramer, W., *Ninth Scientific Report, Imperial Cancer Research Fund*, London, 1930, 21.

⁶ Doerr, R., Bleyer, L., and Schmidt, G. W., *Z. f. Krebsforsch.*, 1932, **30**, 256.

⁷ Sittenfeld, M. J., Johnson, B., and Jobling, J. W., *Am. J. Cancer*, 1932, **16**, 345.

⁸ Mellanby, E., *J. Path. and Bact.*, 1938, **47**, 47.

As a source of the causative agent cultures of filterable fowl sarcoma (strain Rous-Fischer) were employed. This tumor has been maintained in this laboratory for the past 10 years by serial passages *in vitro*. The following design of experiment was adopted: fragments of a Rous cell colony were planted into a Carrel flask containing chicken plasma diluted with Tyrode solution in the proportions of 1:2. After cultivation for 1-3 weeks the flasks were overlaid with Tyrode solution and placed in the incubator for 30 minutes; the supernatant fluid was pipetted off and centrifuged 3 times at 3000 rpm for 15 minutes each time.

Twelve chicks, aged 4 to 10 weeks, in 4 experimental groups were injected with 1 ml of this fluid into the wing vein, 2 or 3 injections being given at weekly intervals to each chick. The fowls succumbed in 21 to 42 days after the initial injection. Necropsy showed new growths in the liver, lungs, heart and kidneys in 9 of the 12 chicks. The tumors in the viscera were always associated with a tumor at the site of the inoculation.

The outstanding feature observed in all chicks was the following: 11 of 12 chicks injected with sarcoma agent displayed most extensive neoplastic bone changes, involving one or several bones (sternum, femur, tibia, tarso-metatarsus, radius, ulna, humerus). Histologically, the tumors were found to be typical osteoid sarcoma destroying the bone and invading the nearby muscles.

The appearance of a general bone neoplasia in a series of animals injected intravenously with Rous agent is noteworthy. This behaviour of Rous agent injected into the blood stream has never been described and in the 10 years during which we have worked with our strain in this laboratory this is the first time that bone tumors appeared either after

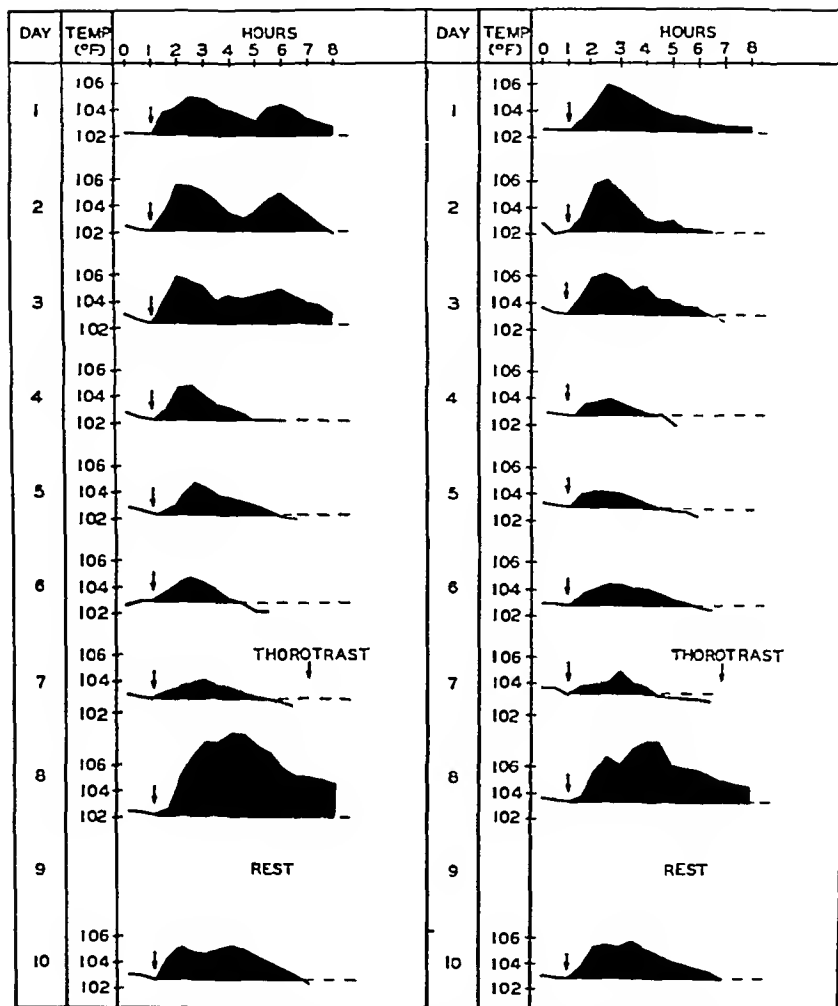


Fig 1

Temperatures of 2 rabbits given daily injections of same dose of typhoid vaccine. After development of tolerance, reticulo endothelial blockade with Thorotrast caused a marked alteration in their temperature responses

the same amount of fever, *i.e.*, a rise of 1.5-2.5°F, with return to the starting temperature in 3 to 5 hours. Four animals received daily injections for the full period of 45 days. After the usual decline in response during the first week these continued to respond to each dose of pyrogen with low grade fevers of about the same extent.

In other experiments longer intervals were allowed to elapse between injections of vaccine. Four rabbits which were injected once a week for 4 months developed some, but

not a very considerable, tolerance. Another group of 4 rabbits received vaccine twice a week. A gradual decline in febrile response occurred during the first 8 to 10 injections, after which they reacted to each dose with about the same degree of fever intermediate between an initial reaction and the minimal reaction noted in animals injected daily. At the end of 4 months the interval was shortened to one day, and they all showed a definite further diminution in their febrile responses.

Reticulo-endothelial blockade produced a

intramuscular or intravenous injections. The cause of this unusual effect of the intravenous inoculation of Rous agent in the experiments reported is entirely obscure. We do not know whether it is to be sought in the particular manner of applying the agent (repeated intravenous injections) or whether we are dealing with a real modification of the agent, which took place spontaneously or resulted from long passage *in vitro*. All these possibilities deserve consideration.

During the preparation of this note a paper of Shrigley, Greene and Duran-Reynolds² ap-

² Shrigley, D. W., Greene, H. S. W. and Duran-Reynolds, F., *Cancer Research*, 1945, 5, 356.

peared. The authors observed that the Rous sarcoma agent after remaining in the anterior chamber of the eye of the guinea pig acquired the ability to produce periosteal tumors in chicks. To judge by the description and the photomicrograph of these growths, they are very similar to those here reported. The acquisition of a new tissue affinity was ascribed to modification of the agent caused by its sojourn in an unnatural environment.

Summary The appearance of an osteoid sarcoma in a series of chickens injected intravenously with the causative agent of Rous sarcoma derived from sarcoma cell cultures, is reported.

15291 P

Development of Tolerance to Typhoid Bacterial Pyrogen and its Abolition by Reticulo-Endothelial Blockade *

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Patients who are being given fever therapy by means of intravenous injections of typhoid vaccine exhibit a decreasing reactivity, and must be given larger and larger doses of the vaccine in order to develop similar fevers at successive treatments. After several injections some individuals may require doses as large as 250 ml of typhoid vaccine in a single day.¹ The mechanism of this remarkable tolerance has not been explained. The present report deals with a study of the phenomenon in rabbits.

The rabbits used were males, of mixed breed, weighing 2 to 3 kg. During test periods they were placed in wooden stalls, and held by head boards. Rectal temperatures were taken at 30-minute intervals, through openings in the floors of the stalls. Observations were never continued for more than 7 hours after giving vaccine, to prevent

undue fatigue of the animals. The vaccine used contained approximately one billion killed *E. typhosa* per ml.[†] The dose was 1 ml of a 1:8 dilution in physiologic salt solution. The agent used for blockade of the reticulo-endothelial system was colloidal thorium dioxide (Thorotrast-Heyden Co.), 9 ml was injected intravenously.

The febrile responses to daily injections of the same dose of vaccine were recorded on 40 rabbits during periods of from 8 to 45 days. The first injection of vaccine always caused a rise in body temperature of 4-5°F, and some fever persisted throughout the 7-hour period of observation. The 2nd and 3rd injections generally caused almost as much fever as the first, but after that there was a decrease in reaction until the 6th to 10th day. Additional injections caused no further diminution, each one inducing approximately

* Aided by a grant from the Venereal Disease Division of the United States Public Health Service.

¹ Heyman, A., *Ten Dis Inform*, 1945, 26, 51.

[†] This vaccine was prepared in the laboratories of the Georgia State Department of Public Health. It is ordinarily used for human immunization and fever therapy.

TABLE I
Average Blood Urea Levels Following Intraperitoneal Injections of Penicillin

Penicillin Oxford units per kg Time after penicillin	No of rats	Blood urea mg per 100 cc				
		0 hr	3 hr	7 hr	11 hr	30 hr
0	10	37	34	29	36	37
50 000*	6	34	32	29	28	30
100 000†	6	41	31	31	31	40
250 000†	6	38	31	—	32	38
500 000†	8	39	36	41	49	40

* Single injection.

† Divided into 5 equal doses Two hours apart

ill for several days

Summary The intraperitoneal injection of penicillin into adult albino rats had no sig-

nificant effect on the blood urea level even when doses as high as 500 000 Oxford units were administered per kg of body weight

15293

Nervous System Mechanism for Epinephrine Secretion

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The rate of liberation of epinephrine from the adrenal glands under ordinary experimental conditions, has been determined as an average of about 0.00025 mg per kg of body weight per minute, in cats and dogs¹. In the absence of experimentally induced alteration of the rate of secretion the range of physiological epinephrine output was found to be approximately 0.0001 to 0.001 mg per kg per minute.

This spontaneous, constant secretion of epinephrine is sustained through the influ-

ence of a centre or centres located in the upper portion of the thoracic division of the spinal cord and can be considered as the normal, physiological secretion. Transection of the cord between the last cervical and the fourth dorsal segments abolishes epinephrine secretion from the adrenals. This centre is bilateral; semisection of the spinal cord, in this region, results in suppression of epinephrine secretion from the ipsilateral adrenal without detectable influence upon the secretion from the contralateral gland².

* Supported by the G. N. Stewart Memorial Fund aided by grants from the Benziehansen and the Sarah Mellon Scaife Foundations.

A preliminary note was published in the *Proc Am Physiol Soc*, 1931. The study was interrupted when the Department of Experimental Medicine, at Western Reserve University, was discontinued. Some of the experiments were performed at the University of Chicago. The investigation was resumed and continued in this laboratory.

¹ Stewart, G. N. and Rogoff, J. M., a *J Pharm and Exp Therap*, 1916, 8, 479, b *Am J Physiol*, 1923, 66, 235.

Incidental observations made in the course of some other investigations suggested the probability of the existence of an inhibitory centre for epinephrine secretion located in the brain. For example the large increase in epinephrine output which is induced by the action of strychnine usually is preceded by a preliminary decline in the rate of secretion if a minimal effective dose of the drug is injected intravenously, or a larger dose

² Stewart, G. N. and Rogoff, J. M., a *J Exper Med*, 1917, 26, 613, b *Am J Physiol*, 1920, 31, 484.

striking effect on the temperature responses of rabbits that had been previously "trained" by repeated daily injections of vaccine. A typical result is illustrated in Fig 1. Here it will be noted that a considerable modification in response was manifest by the 7th day. Each animal was then given Thorotrast. On the following day administration of typhoid vaccine caused high, prolonged temperature elevations. After a rest of one day their febrile responses to the vaccine were again considerably lessened. This brief effect of the blocking agent is in line with other experience on functional interference with the reticulo-endothelial system.²

² Jaffe, R. H., *Physiol Rev*, 1931, 11, 277

These experiments show that rabbits can develop a tolerance to typhoid bacterial pyrogen, and that the tolerance is most marked when injections are given frequently. The mechanism of development of this state is not yet explained. Certain other observations, which cannot be given in detail here, indicate that the production of specific humoral antibodies is not responsible, and furthermore, that the development of tolerance to typhoid vaccine carries with it a similar alteration in response to other bacterial pyrogens. Possibly the process involves a change in the functional activity of the reticulo-endothelial system, providing for more rapid disposal of the foreign material.

15292

Effect of Penicillin on Blood Urea in the Rat

JACK RALPH LEONARDS AND FLORENCE WILLIAMS
(Introduced by Victor C. Myers)

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Early in 1943 the Floreys¹ reported that 4 out of 5 patients treated with 120,000 or more Oxford units of penicillin manifested a rise in blood urea. The elevated values returned rapidly to normal when the drug was withdrawn. Although no explanation was advanced for this phenomenon it was not thought to be due to renal damage.

The present study demonstrates that penicillin has no significant effect on the blood urea level of normal rats even though exceedingly large amounts of the drug are administered.

The penicillin employed was a rather impure preparation assaying 300 Oxford units per mg of total solids.* Normal adult albino rats of both sexes were used and the penicillin

was injected intraperitoneally in a solution containing 25,000 Oxford units per ml. Samples of blood (0.1 or 0.2 ml) for urea determinations were obtained from the tail vein directly into a pipette and the urea was analyzed by the colorimetric method described by Ormsby.²

The results are presented in Table I. It is immediately evident that no significant increase in blood urea results even after the administration of 500,000 Oxford units per kg of body weight. This dosage represents the largest amount of our preparation of penicillin that could be tolerated by the rat without any apparent toxic manifestations. It should be emphasized that this is a tremendously large dose. When a group of rats were given 1,000,000 Oxford units of penicillin per kg of body weight over a period of 10 hours, 4 of the 6 rats died on the same day and the 2 surviving animals were severely

¹ Florey, M. E., and Florey, H. W., *Lancet*, 1943, 1, 387

* Kindly supplied by Ben Venue Laboratories, Inc., Bedford, Ohio

² Ormsby, A. A., *J. Biol. Chem.*, 1942, 146, 595

TABLE I
Epinephrine Output from the Adrenal after Decapitation *

		Before brain section					After brain section				
No	wt. kg	Blood pressure mm Hg	Blood flow μ /min	Epinephrine		Infus. vol. ml	Blood pressure mm Hg	Blood flow μ /min	Epinephrine		
				Concentration $\times 100,000$	Output μ g/kg/min				Concentration $\times 100,000$	Output μ g/kg/min	
1	3.0		2.32	1.64	0.11	10		2.31	1.21	1.1	
2	2.9	86	1.36	1.32	0.12	12	90	1.97	1.83	2.25	
3	2.9	120	1.28	1.58	0.2	5	78	2.15	1.85	2.88	
4	2.7	91	2.52	1.80	0.11	15	10	0.62	1.10	0.21	
5	3.2	156	7.09	1.52	0.13	5	96	5.8	1.20	2.9	
6	3.2	116	6.2	1.16	0.12	10	68	2.25	1.65	1.16	
7	3.3	154	6.8	1.33	0.26	8	11	3.07	1.11	2.16	
8	2.3	112	1.0	1.35	0.86	8	38	0.51	1.10	0.51	
9	2.1	120	5.2	1.125	1.22	10	72	2.88	1.75	1.81	
10	2.7	97	1.1	1.51	0.81	9	52	2.7	1.70	1.86	
11	2.7	74	2.08	1.325	0.61	4	58	1.57	1.90	1.72	
12	3.1	150	2.7	1.15	0.6	6	77	1.0	1.12	2.18	
13	2.8	85	1.2	1.175	0.88	5	88	3.5	1.11	1.1	
14	2.7	102	7.1	1.90	0.79	7	93	7.0	1.21	1.11	
15	3.1	118	3.8	1.05	0.58	1	11	0.16	1.12	1.1	
16	2.8	90	2.6	1.30	0.87	6	90	2.51	1.875	2.97	
17	2.6	105	1.74	1.21	0.81	15	36	0.15	1.10	0.15	
18	2.7	150	1.11	1.51	0.83	10	81	2.18	1.375	0.66	
19	2.3	125	1.6	1.70	0.66	7	61	2.0	1.31	0.61	
20	2.2	90	3.1	1.10	1.04	8	68	1.3	1.97	1.33	
21	2.9	115	5.9	1.55	1.08	5	126	1.4	1.35	1.26	
22	3.2	122	6.11	1.50	1.11	5	81	2.93	1.225	1.3	
23	2.1	136	6.2	1.55	1.13	1	97	3.82	1.11	1.16	
24	3.7	112	4.4	1.37	1.19	5	88	2.1	1.70	0.99	
25	3.1	130	2.10	1.25	0.88	5	66	1.01	1.128	0.79	
26	2.9	122	1.8	1.10	0.95	4	70	1.15	1.075	1.18	
27	3.0	110	3.2	1.24	1.11	6	82	2.6	1.20	1.15	
28	2.5	122	1.11	1.52	0.66	6	97	2.86	1.10	0.71	

* In cats 116, metabolic decapitation was between the superior colliculi and the optic chiasm, in all the others, it was anterior to that level.

subcutaneously Intravenous administration of the larger doses generally causes only the augmentation of epinephrine output³ This suggests a possible activity of an inhibitory mechanism during the phase of cerebral stimulation by strychnine Such an inhibitory influence upon epinephrine secretion would be submerged by the more powerful action of the opposing spinal cord centre, when the predominant influence of strychnine on the cord is effected

Other observations likewise indicated the possibility that an inhibitory centre, which influences epinephrine secretion, exists in the brain For example, when experimental animals were rendered insensitive to pain by compression or destruction of the brain, after preliminary anesthesia, the epinephrine output from the adrenal glands was found to be within but near or at the maximum of the range of spontaneous liberation^{2a, 8} Furthermore, the same observation was made when the rate of liberation of epinephrine was determined in animals with cerebral anemia induced by occlusion of the blood supply to the brain⁴

In the present series of experiments, the rate of epinephrine secretion from the adrenal glands was determined before and after transection at various levels of the brain Decerebration was performed with the special knife described by Karrer and Stevens⁵ All of the experiments were performed on cats The animals were anesthetized with urethane administered by stomach tube The results obtained are illustrated in Table I and Fig 3

The level of decerebration was verified at the end of each experiment, by post-mortem examination In Experiments 1-16, inclusive, transection of the brain was at levels bounded by the superior colliculus and the optic chiasm In the others, decerebration was at various levels anterior to this In 6 of the 28 animals, a small area of cortex escaped section (No 7, 17, 18, 20, 24, 26)

The rate of secretion of epinephrine was assayed by the method of Stewart and Rogoff Adrenal blood specimens were collected via the "cava pocket," before and after transection of the brain The amount of blood obtained and the duration of the collection were measured, thus determining the rate of blood flow from the adrenals The concentration of epinephrine in the blood specimens was assayed by the quantitative inhibition of rabbits' intestine segments From this information, the rate of epinephrine secretion was readily calculated In a few of the experiments, assay of the adrenal blood specimens was confirmed employing the "sensitized eye" method⁶

Table I illustrates the decided increase in the rate of liberation of epinephrine which follows transection of the brain between the superior colliculus and the optic chiasm (cats 1-16, inclusive) In 12 of the animals the epinephrine output rose to or near the maximum of the normal range of spontaneous liberation In cats 1 and 15, the output did not approach the upper limit of the range, although there was an increase of 2 and 3 times the initial rate of epinephrine secretion

In one cat (8) there was, if anything, a slight reduction in the output The adrenal blood flow, following decerebration, was very low in cats 4, 8 and 15 Brain transection was associated with excessive hemorrhage in these animals However, experimental shock due to loss of systemic blood was not found to alter the epinephrine output from the adrenal glands⁷

The 12 experiments in which transection of the brain was performed through or anterior to the optic chiasm showed no significant alteration of the rate of liberation of epinephrine from the adrenals, except in cat 24 In this animal, a reduction to about one-fourth of the initial epinephrine output was found, although the blood flow from the adrenals was good A reduction in output to about one-half was found in cat 17 but

³ Stewart, G N, and Rogoff, J M, *J Pharm and Exp Therap*, 1919, **13**, 95

⁴ Rogoff, J M, *Am J Physiol*, 1924, **67**, 551

⁵ Karrer, E, and Stevens, H C, *J Lab Clin Med*, 1928, **14**, 266

⁶ Rogoff, J M, *Proc Soc Exp Biol and Med*, 1937, **36**, 441

⁷ Stewart, G N, and Rogoff, J M, *Am J Physiol* 1919, **48**, 22

TABLE I
Epinephrine Output from the Adrenals after Decerebration *

Before brain section						After brain section					
No	wt kg	Epinephrine				Inter val min	Blood pressure mm Hg	Blood flow g/min	Epinephrine		Output $\mu\text{g/kg/min}$
		Concentration $\times 100,000$	Output $\mu\text{g/min}$	Concentration $\times 100,000$	Output $\mu\text{g/min}$						
1	3.0		2.32	1.64	0.33	10		2.31	1.21	1.1	0.37
2	2.9	86	1.36	1.12	0.42	12	90	1.87	1.83	2.25	0.8
3	2.9	120	3.28	1.38	0.57	5	78	2.15	1.85	2.88	1.0
4	2.7	93	2.52	1.80	0.31	15	40	0.62	1.10	0.21	0.09
5	3.2	156	7.09	1.52	1.37	5	96	5.8	1.20	2.9	0.91
6	3.2	140	6.2	1.46	1.35	10	68	2.25	1.65	3.46	1.08
7	3.3	154	6.8	1.33	2.06	8	44	1.07	1.43	2.19	0.75
8	2.3	142	3.0	1.35	0.86	8	38	0.54	1.10	0.71	0.23
9	2.1	120	5.2	1.425	1.22	10	72	2.88	1.75	3.81	1.6
10	2.7	97	4.3	1.51	0.84	8	52	2.7	1.70	3.86	1.13
11	2.7	74	2.08	1.325	0.64	1	78	1.55	1.90	1.72	0.64
12	3.1	150	2.7	1.45	0.6	1	88	1.0	1.42	2.38	0.77
13	2.8	85	4.2	1.475	0.88	5	93	1.7	1.11	4.1	1.46
14	2.7	102	7.1	1.90	0.79	7	93	7.0	1.21	3.33	1.23
15	3.3	118	3.8	1.65	0.78	1	44	0.46	1.42	1.1	0.33
16	2.8	96	2.6	1.70	0.87	6	90	2.51	1.875	2.97	1.03
17	2.6	95	1.74	1.21	0.83	15	36	0.45	1.10	0.45	0.17
18	2.7	150	4.41	1.53	0.83	10	84	2.15	1.375	0.66	0.24
19	2.3	125	4.6	1.70	0.66	7	64	2.0	1.31	0.64	0.28
20	2.2	90	3.1	1.70	1.03	5	68	1.3	1.97	1.71	0.61
21	2.9	145	7.9	1.55	1.08	5	126	3.4	1.35	1.26	0.41
22	3.2	122	6.41	1.56	1.14	5	84	2.93	1.225	1.3	0.41
23	2.4	130	6.2	1.55	1.13	4	95	3.82	1.33	1.36	0.48
24	3.7	112	4.4	1.37	1.19	5	88	2.4	1.70	0.34	0.09
25	3.1	130	2.16	1.25	0.86	5	66	1.01	1.128	0.79	0.25
26	2.9	122	3.8	1.40	0.95	4	70	1.15	1.975	1.18	0.41
27	3.9	110	3.2	1.28	1.14	6	82	2.9	1.20	1.47	0.37
28	2.5	122	3.41	1.52	0.66	6	97	2.86	1.40	0.71	0.28

* In cats 1-16, inclusive, decerebration was between the superior colliculi and the optic chiasm, in all the others, it was anterior to that level.

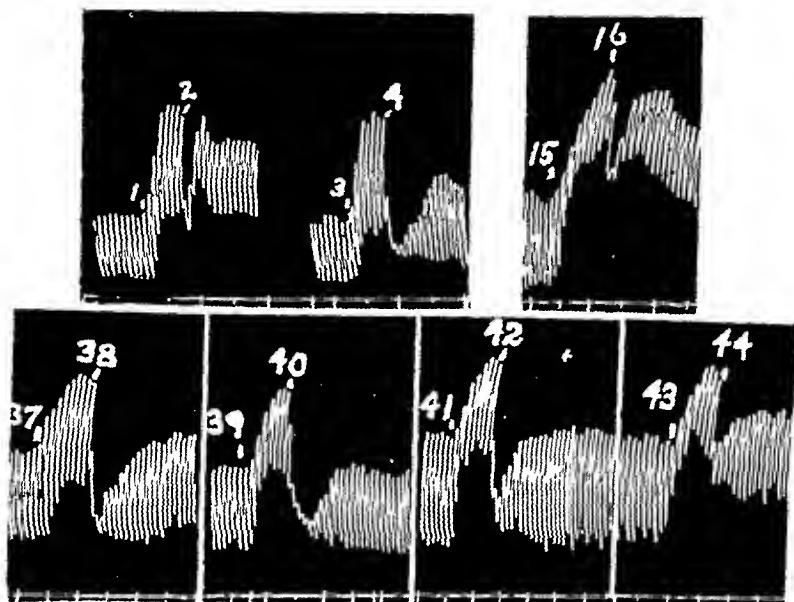


Fig 1

At the uneven numbers Ringer's solution was displaced by indifferent blood and this at 2 by adrenal blood specimen A, at 4, 38 and 42 by adrenal blood specimen B, at 16 by indifferent blood to which was added adrenalin to make a concentration of 1 7,000,000, at 40 a concentration of 1 1,450 000, and at 44 a concentration of 1 2,850 000 A was assayed at 1 6,400,000, and B at 1 2,100,000 Time in half minutes Reduced to two thirds

in this case, as in cats 4, 8 and 15 the adrenal blood flow was very slow. The question may be raised, in these cases, whether trauma of the brain, in a region near the inhibitory centre for epinephrine secretion might not result in stimulation of that centre and consequent reduction of epinephrine output from the adrenal glands.

In 2 experiments (cats 3 and 5), in which the decerebration was between the superior colliculus and the optic chiasm, and in one (cat 19), in which the decerebration was anterior to this region, additional adrenal blood specimens were obtained for assay. In cat 3, a specimen obtained 25 minutes after decerebration, when the adrenal blood flow was 115 g per minute, was assayed at 1 450,000 adrenalin, corresponding to an output of 0.0025 mg per minute for the cat or 0.00086 mg per kg per minute. In cat 5, a specimen obtained 18 minutes after decerebration, when the adrenal blood flow was 1.02 g per minute, was assayed at 1 750,000 adrenalin, corresponding to an output of

0.00136 mg per minute for the cat or 0.00048 mg per kg per minute. In the case of transection of the brain through the optic chiasm (cat 19), a specimen obtained 35 minutes after decerebration, when the adrenal blood flow was 1.4 g per minute showed the same epinephrine output from the adrenals as before and 7 minutes after the decerebration.

To illustrate the results obtained, condensed protocols and some of the tracings (Fig 1, 2) from the assay in 2 of the experiments are given (cats 1 and 13). In these animals the transection of the brain was between the superior colliculi and the optic chiasm. Cat 1 was a pregnant female and cat 13 a male. These animals were selected for illustration because the results are unequivocal. The rate of blood flow from the adrenal glands did not change after decerebration, nevertheless, the epinephrine concentration in the adrenal blood specimens obtained after the decerebration (B) was decidedly greater than that in the initial specimens (A).

Condensed protocol Cat 1 pregnant fe-

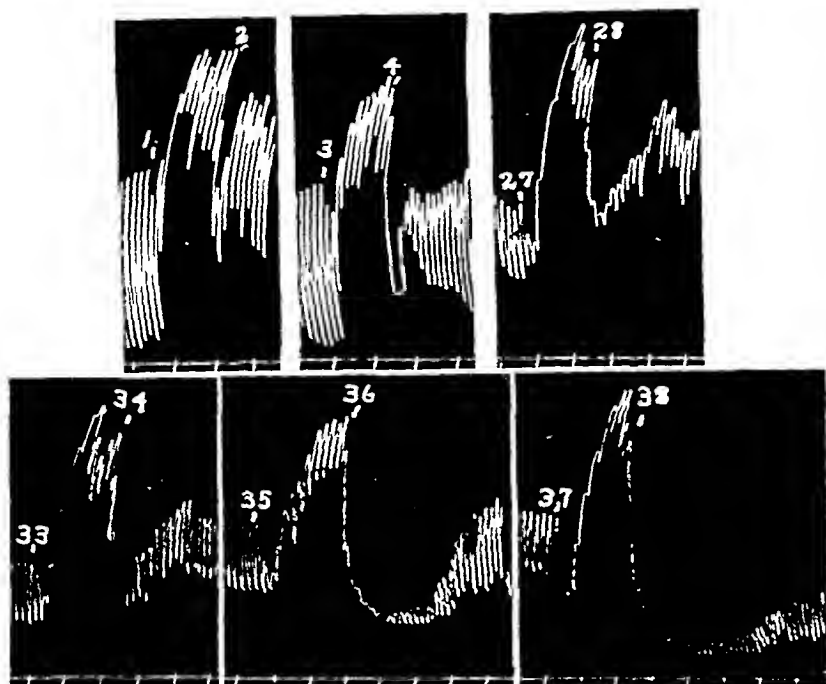


Fig 2

At the uneven numbers Ringer's solution was displaced by indifferent blood and thus at 2 and 34 by adrenal blood specimen A, at 4 and 36 by adrenal blood specimen B, at 28 by indifferent blood to which was added adrenalin to make a concentration of 1 6,250,000, and at 38 a concentration of 1 700 000. A was assayed at 1 4,750 000 and B at 1 1100,000. Time in half minutes. Reduced to three fifths.

male, weight 30 kg

a m

8 45, Urethane (20 cc of 20% sol) by stomach tube

10 45, Anesthesia complete

11 22, Collected adrenal blood specimen A
58 g in 2½ min, blood flow, 232 g per minute

11 40, Inserted tracheal cannula and trephined skull

11 42, Decerebration

11 52, Collected adrenal blood specimen B,
578 g in 2½ min, blood flow, 231 g per minute

Complete transection of the brain, just anterior to the superior colliculi, verified by post mortem examination

Specimen A assayed at 1 6,400,000 adrenalin, corresponding to an output of 0 00033 mg per minute for the cat or 0 00011 mg per kg body weight per minute. Specimen B, assayed at 1 2,100,000 adrenalin, corresponding to an output of 0 0011 mg per minute for the cat or 0 00037 mg per kg body weight per minute (Fig 1)

men B, assayed at 1 2,100,000 adrenalin, corresponding to an output of 0 0011 mg per minute for the cat or 0 00037 mg per kg body weight per minute (Fig 1)

Condensed protocol Cat 13, male, weight, 28 kg

a m

9 30 Urethane (25 cc of 20% sol) by stomach tube

10 30 Anesthesia complete

10 55 Collected adrenal blood specimen A,
84 g in 2 min blood flow, 42 g per minute

11 12, Inserted tracheal cannula and trephined skull

11 18 Decerebration

11 23 Collected adrenal blood specimen B,
90 g in 2 min blood flow, 45 g per minute

Complete transection of the brain, about ⅓ inch anterior to the superior colliculi, verified

TABLE II
Statistical Summary of Epinephrine Output

Cats	Decerebration	Me in $\mu\text{g/kg/min}$	Squared standard deviation	Probable error of mean	Ratio of std error of diff to diff of means		t	
					Observed	Significant	Observed	Significant
1 16	before	0.296	0.146	0.025				
1 16	after	0.858	0.451	0.076	7.02	3.0	4.736	2.131
17 28	before	0.339	0.069	0.013				
17 28	after	0.335	0.144	0.028	0.031	3.0	0.891	2.201
1 16	before	0.296	0.146	0.025				
17 28	before	0.858	0.069	0.013	1.529	3.0	0.939	2.201

by post mortem examination

Specimen A, assayed at 14,750,000 adrenalin, corresponding to an output of 0.00088 mg per minute for the cat or 0.00031 mg per kg body weight per minute. Specimen B, assayed at 11,100,000 adrenalin, corresponding to an output of 0.0041 mg per

minute for the cat or 0.00146 mg per kg body weight per minute (Fig. 2)

Although the results are sufficiently definite to support our conclusions, they are presented in the following statistical summary (Table II) for which we are greatly indebted to Dr. Paul L. McLain of the Department of

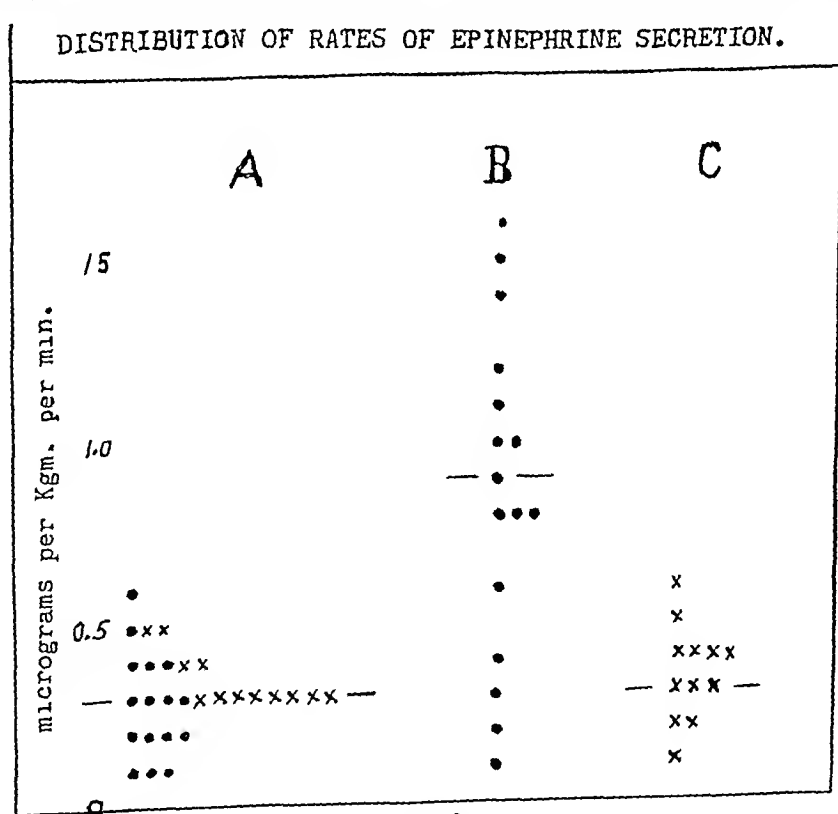


Fig. 3

A—Before decerebration
B—After decerebration between superior colliculi and optic chiasm
C—After decerebration anterior to level of B
●—Cats 1 16, inclusive
X—Cats 17 28, inclusive

Physiology The distribution of the rates of epinephrine output is illustrated in Fig 3

Summary These experiments indicate the existence of an inhibitory central mechanism, located in the region of the brain bounded by the superior colliculi and the optic chiasm, which influences epinephrine secretion from the adrenal glands. The existence of a central mechanism in the spinal cord, which exercises an opposite influence to that of this cerebral centre, was established earlier.² Removal of the influence of the brain centre, results in epinephrine output from the adrenals at the maximum rate of spontaneous liberation which can be sustained through the physiological activity of the spinal cord centre. This rate corresponds with the upper limit of the range of normal epinephrine secretion.¹

Despite the fact that epinephrine was the first hormone to be isolated from its source, and to be synthesized, the functional significance of epinephrine secretion is still obscure. Physiological interpretations from pharmacological observations often have been misleading. Only quantitative experiments, yielding results within the limits of the

physiological rate of liberation, can be expected to yield unequivocal information on the function of epinephrine secretion.

In the light of the experiments reported herewith it can be seen why reflex augmentation of the rate of epinephrine secretion has not been demonstrated successfully in animals under the influence of asphyxia³ or stimulation of sensory nerve trunks.⁴ For, even if these stimuli might affect appropriate receptors, which could alter the rate of epinephrine secretion, there is no reason to assume that they might not equally influence the cerebral and the cord centres, thus defeating the possibility of eliciting reflexly either augmentation or diminution in the rate of epinephrine secretion from the adrenal glands. In preliminary tests, it was not found possible to elicit augmentation of the epinephrine output by stimulation of sensory nerves, following mechanical destruction or compression of the brain.^{5a}

³ Stewart, G. N., and Rogoff, J. M., *J. Pharm. and Exp. Therap.*, 1917, 10, 49.

⁴ Stewart, G. N., and Rogoff, J. M., a) *J. Exper. Med.*, 1917, 26, 637, b) *Am. J. Physiol.*, 1924, 69, 605.

15294 P

Inhibition of Experimental Drug Allergy by Prior Feeding of the Sensitizing Agent

MERRILL W. CHASE

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In the course of studies on experimental drug allergy, it was noticed that sensitization was apt to be less successful in guinea pigs which previously had been treated briefly with the same chemical. Experiments were undertaken to establish at will such a refractory state.* For the preparatory, or

blocking, treatment, the methods tried included the parenteral injection of soluble and insoluble protein conjugates of the incitant itself, repeated cutaneous application of the chemicals in very low (non-sensitizing) concentration, intravenous injections, and feeding experiments. After this preliminary treatment, a rest period of 2 weeks was allowed, and then the animals, in parallel with fresh guinea pigs as controls, were subjected to an active sensitizing course consisting of 6 or more intracutaneous injections of the incitant over a period of several weeks. Following

* In the case of neorphenamine, Sulzberger had found that a single intravenous injection of the substance usually prevented the active sensitization sought by an intracutaneous injection made one day previously.

TABLE I

Hypersensitivity rated as	Prior feeding of allergen (93 animals)	Controls (77 animals)
	%	%
High	3.2	74.0
Good	0.0	16.9
Moderate	8.6	5.2
Weak	20.4	3.9
Low	46.2	0.0
Very faint or entirely negative	21.5	0.0

another 2 weeks' rest, the outcome of the challenging sensitization was determined by a "contact test" in which the skin was painted with dilutions of the incitant in olive oil and the animals were examined for the development of cutaneous reactions.

While several substances were employed in the first experiments, the compound 2,4-dinitrochlorobenzene was chosen for chief attention because it is actively allergenic and is well known in the experimental sensitization of human beings.^{1,2,3} Rather unexpectedly, and in sharp (but not absolute) contrast to all other methods employed, a blocking effect of substantial degree was found to be induced by feeding the chemical. A 1% solution of dinitrochlorobenzene in olive oil was fed from the tip of a pipette in such manner that contact of the solution with the muzzle was minimal. The feedings (0.3 cc) were made daily for 6 days, followed by an 8-day rest period, 2 or 3 such courses were given prior to the attempt at sensitization by intracutaneous injections. While the latter regularly sensitized either normal animals or animals fed the vehicle alone (olive oil), a very considerable diminution in effect was encountered in groups that had received prior feedings of the chemical. This is shown in Table I, which brings together the results from various, essentially similar experiments. It will be seen that the protection afforded was not absolute, although in some experi-

ments well-nigh complete inhibition was attained. On the other hand, it remains possible that the protective effect may be overridden by intensive treatment with the allergen.

To examine whether the feedings had led to a blocking mechanism directed only towards the same chemical or had induced a non-specific resistance to sensitizations, the behavior of the animals to an active sensitizing course with a second, unrelated substance (*o*-chlorobenzoyl chloride) was examined. The resistant and "positive control" groups did not differ at all in their response to the new agent. This held true, also, when animals fed with one chemical then received a simultaneous series of intracutaneous injections with 2 substances, one, the specific allergen, and the other, a non-related compound. The inhibitory effect therefore was specific, and the animals were normal in their response to another compound.

That the effect is rather durable, and one apparently not dependent upon retention of the ingested substance, was demonstrated in one lot of 30 animals fed 2,4-dinitrochlorobenzene. At varying intervals, groups of approximately 10 animals were given the sensitizing course, along with an equal number of new animals as controls. Protection was still apparent even in the last group tested here the interval between the final feeding and the beginning of the challenging course had been 27 weeks, and the final contact test was made 4 weeks later.

It is possible, then, to induce a profound and lasting protection against experimental sensitization when one starts with animals which are not already sensitive to the substance in question, this is consonant with the experiment of Sulzberger.⁴ On the other hand, courses of feeding given to animals previously made sensitive to 2,4-dinitrochlorobenzene have so far shown no appreciable effect in diminishing the degree of hypersensitivity, a result in accord with attempts to decrease an established sensitivity in guinea pigs by parenteral injection of soluble protein

¹ Wedroff, N. S., and Dolgoff, A. P., *Arch. Derm. & Syph.*, 1935, **171**, 647.

² Lundsteiner, K., Rostenberg, A., and Sulzberger, M. B., *J. Invest. Dermat.*, 1939, **2**, 23.

³ Hartman, H., *Acta Dermat. Venereol.*, 1939, **20**, 257.

⁴ Sulzberger, M. B., *Arch. Derm. and Syph.*, 1930, **22**, 839.

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⁶Landsteiner, K. and Chase, M. W. *J. Exp. Med.* 1937 **60** 337.

⁷Park, R. G. *Brit. Med. J.* 1944 **2** 810.

Summary Through the feeding of certain allergenic compounds to the non-sensitive subject, a state of resistance may be established against subsequent experimental sensitization of the skin by the same substance.

⁷Stevens, F. A. *J. Am. Med. Assn.*, 1945, **127**, 912.

15295

Rabbit Papilloma and Vaccinia Viruses and T₂ Bacteriophage of *E. coli* in "Shadow" Electron Micrographs *

D. G. SHARP, A. R. TAYLOR, A. E. HOOK, AND J. W. BEARD †

From the Department of Surgery, Duke University School of Medicine, Durham, N. C.

Although electron microscopy has added greatly to knowledge of the shape, size and other characters of viruses, the findings leave much to be desired. The capacity of viruses to absorb and scatter fast electrons is relatively small, and consequently, images of the particles are likely to be of low contrast and indistinct, especially at the periphery. Contrast in influenza virus images may be enhanced slightly by treatment of the virus with osmic acid,¹ use of calcium chloride, though obscuring internal structure aids greatly in microscopy of equine encephalomyelitis² and influenza viruses,³ but

not in the instances of the rabbit papilloma and tobacco mosaic viruses. In addition, the images are flat, and judgment of particle shape must be based solely on contour portrayed in 2 dimensions. A considerable advance in the electron microscopy of viruses is the application of the shadow-casting technique recently reported by Williams and Wyckoff.⁴⁻⁶ In this process, the molecules of metals evaporated *in vacuo* fall on the virus particles at a grazing angle and cast "shadows" which give the appearance of 3-dimensional contour to images in electron micrographs. The shape and length of the shadows cast on the background and the contour of the images afford a much better basis for judgment of the shape of the particle than the unshadowed images. Studies with this technique have been made of the vaccinia and rabbit papilloma viruses and the T₂ bacteriophage of *E. coli*. The results⁷ with these 3 viruses are reported in the present paper.

Materials and Methods The viruses were

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⁴Williams, R. C. and Wyckoff, R. W. G. *Proc. Soc. Exp. Biol. and Med.* 1945 **58** 265.

⁵Williams, R. C. and Wyckoff, R. W. G., *Nature* 1945 **156** 68.

⁶Williams, R. C., and Wyckoff, R. W. G.,

* This work was aided by the Commission on Influenza and the Commission on Epidemiology, Survey Board for the Investigation and Control of Influenza and Other Epidemic Diseases in the Army, Preventive Medicine Service Office of the Surgeon General, United States Army, and by a grant to Duke University from Lederle Laboratories, Pearl River, New York.

† Consultant to Secretary of War and Member Commission on Acute Respiratory Diseases Board for the Control of Influenza and Other Epidemic Diseases in the Army, Preventive Medicine Service Office of the Surgeon General, United States Army.

¹Sharp, D. G., Taylor, A. R., McLean, I. W., Jr., Beard, D., Beard, J. W., Feller, A. E. and Dingle, J. H. *J. Immunol.* 1944 **48** 129.

²Sharp, D. G., Taylor, A. R., Beard, D. and

TABLE I

Hypersensitivity rated as	Prior feeding of allergen (93 animals)	Controls (77 animals)
	%	%
High	3.2	74.0
Good	0.0	16.9
Moderate	8.6	5.2
Weak	20.4	3.9
Low	46.2	0.0
Very faint or entirely negative	21.5	0.0

another 2 weeks' rest, the outcome of the challenging sensitization was determined by a "contact test" in which the skin was painted with dilutions of the incitant in olive oil and the animals were examined for the development of cutaneous reactions.

While several substances were employed in the first experiments, the compound 2,4-dinitrochlorobenzene was chosen for chief attention because it is actively allergenic and is well known in the experimental sensitization of human beings.^{1,2,3} Rather unexpectedly, and in sharp (but not absolute) contrast to all other methods employed, a blocking effect of substantial degree was found to be induced by feeding the chemical. A 1% solution of dinitrochlorobenzene in olive oil was fed from the tip of a pipette in such manner that contact of the solution with the muzzle was minimal. The feedings (0.3 cc) were made daily for 6 days, followed by an 8-day rest period, 2 or 3 such courses were given prior to the attempt at sensitization by intracutaneous injections. While the latter regularly sensitized either normal animals or animals fed the vehicle alone (olive oil), a very considerable diminution in effect was encountered in groups that had received prior feedings of the chemical. This is shown in Table I, which brings together the results from various, essentially similar experiments. It will be seen that the protection afforded was not absolute, although in some experi-

ments well-nigh complete inhibition was attained. On the other hand, it remains possible that the protective effect may be overridden by intensive treatment with the allergen.

To examine whether the feedings had led to a blocking mechanism directed only towards the same chemical or had induced a non-specific resistance to sensitizations, the behavior of the animals to an active sensitizing course with a second, unrelated substance (*o*-chlorobenzoyl chloride) was examined. The resistant and "positive control" groups did not differ at all in their response to the new agent. This held true, also, when animals fed with one chemical then received a simultaneous series of intracutaneous injections with 2 substances, one, the specific allergen, and the other, a non-related compound. The inhibitory effect therefore was specific, and the animals were normal in their response to another compound.

That the effect is rather durable, and one apparently not dependent upon retention of the ingested substance, was demonstrated in one lot of 30 animals fed 2,4-dinitrochlorobenzene. At varying intervals, groups of approximately 10 animals were given the sensitizing course, along with an equal number of new animals as controls. Protection was still apparent even in the last group tested here the interval between the final feeding and the beginning of the challenging course had been 27 weeks, and the final contact test was made 4 weeks later.

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² Landsteiner, K., Rostenberg, A., and Sulzberger, M. B., *J. Invest. Dermat.*, 1939, **2**, 25.

³ Haxthausen, H., *Acta Dermatol. Venereol.*, 1939, **20**, 257.

⁴ Sulzberger, M. B., *Arch. Derm. and Syph.*, 1930, **22**, 839.

conjugates of the incitant.⁷ There are, however, statements in the literature to the effect that sensitive human beings have been partially desensitized by ingestion of the specific allergen.^{6,7}

⁷ Landstamer, K. and Chase, M. W., *J. Exp. Med.* 1937 **66** 337.

⁶ Park, R. G., *Brit. Med. J.* 1944 **2** 816.

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⁷ Stevens, F. A., *J. Am. Med. Assn.*, 1945, **127**, 912.

15295

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Although electron microscopy has added greatly to knowledge of the shape, size and other characters of viruses, the findings leave much to be desired. The capacity of viruses to absorb and scatter fast electrons is relatively small, and, consequently, images of the particles are likely to be of low contrast and indistinct especially at the periphery. Contrast in influenza virus images may be enhanced slightly by treatment of the virus with osmic acid,¹ use of calcium chloride, though obscuring internal structure, aids greatly in micrographs of equine encephalomyelitis² and influenza viruses³ but

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¹ Sharp, D. G., Taylor, A. R., McLern, I. W., Jr., Beard, D. Beard, J. W., Feller, A. E., and Dingle, J. H., *J. Immunol.*, 1944, **48**, 129.

² Sharp, D. G., Taylor, A. R., Beard, D. and

not in the instances of the rabbit papilloma and tobacco mosaic viruses. In addition, the images are flat, and judgment of particle shape must be based solely on contour portrayed in 2 dimensions. A considerable advance in the electron microscopy of viruses is the application of the shadow-casting technique recently reported by Williams and Wyckoff.^{4,5} In this process, the molecules of metals evaporated *in vacuo* fall on the virus particles at a grazing angle and cast "shadows" which give the appearance of 3-dimensional contour to images in electron micrographs. The shape and length of the shadows cast on the background and the contour of the images afford a much better basis for judgment of the shape of the particle than the unshadowed images. Studies with this technique have been made of the vaccinia and rabbit papilloma viruses and the T₂ bacteriophage of *E. coli*. The results[†] with these 3 viruses are reported in the present paper.

Materials and Methods The viruses were

Beard, J. W., *Arch. Path.* 1943 **36**, 167.

³ Taylor, A. R., Sharp, D. G., Beard, D. Beard, J. W., Dingle, J. H. and Feller, A. E., *J. Immunol.*, 1943 **47**, 261.

⁴ Williams, R. C., and Wyckoff, R. W. G., *Proc. Soc. Exp. Biol. and Med.*, 1945, **58**, 265.

⁵ Williams, R. C. and Wyckoff, R. W. G., *Nature*, 1945 **156**, 68.

⁶ Williams, R. C., and Wyckoff, R. W. G.,

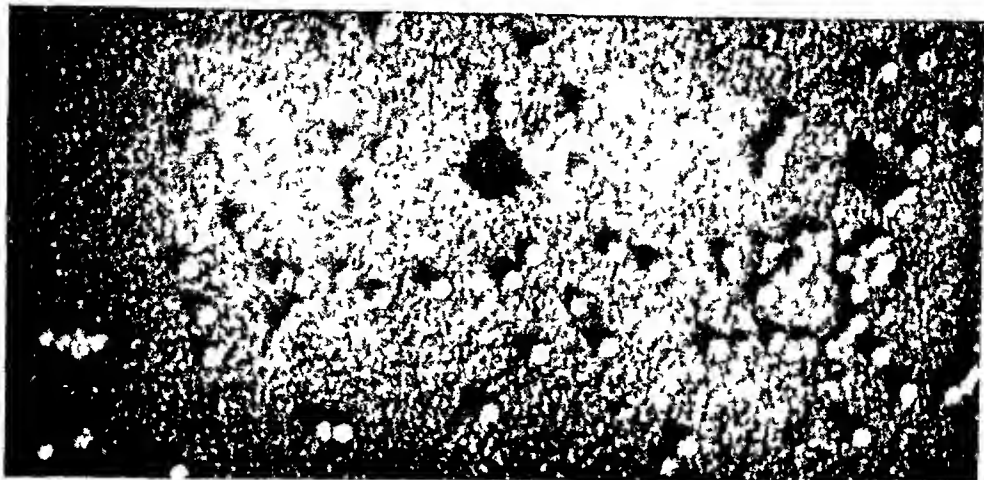


Fig 1

Rabbit papilloma virus shadowed with 10 mg of gold at the angle tangent $2/7$ Magnification 42,700 \times

employed only in purified preparations *Vaccinia*^{7,8} and rabbit papilloma viruses^{9,10} were obtained as previously described. The bacteriophage,⁶ the T_2 strain¹¹ parasitizing *E. coli*, was cultivated on the host grown in broth and purified by ultracentrifugal procedures which will be described elsewhere.¹² Purified virus suspensions containing about 10^{12} particles per ml were placed on the collodion film support, and, after about half a minute, the excess fluid was removed with a fine pipette. The films after drying were viewed immediately in the electron microscope or, more often, lightly washed by flood-

ing once or twice with or dipping in distilled water. When the films were again dry, they were ready for observation and electron micrography in the ordinary way, or for application of evaporated metal. In some instances the films were observed and photographed, then shadowed and examined again.

Gold was used for the papilloma and vaccinia viruses and chromium for the bacteriophage. The weighed metals were evaporated in a 10-turn conical helix of 5 mil tungsten wire, through which alternating current was passed from a 500-volt-ampere transformer at 20 volts. Enough heat was generated to complete the evaporation in 1 to 3 minutes. The vacuum chamber was a glass tube 1.5 inches in diameter and 12 inches long. It was continuously evacuated with a Cenco Hivac pump and a 2-stage mercury diffusion pump, giving an ultimate vacuum of the order of 10^{-5} mm of Hg. The metal was deposited on the papilloma and vaccinia viruses at the angle whose tangent was $2/7$ and on the bacteriophage at the angle of tangent $1/5$. The distance from the evaporator coil to the virus was 7 cm. Assuming evaporation equally in all directions, the metal thickness on the side of the virus particle normal to the beam would be given by the equation

$$\text{Thickness} = \frac{W}{4\pi r^2 \rho}$$

Science, 1945, **101**, 596

⁴ This work was described in part at the meeting of the Electron Microscope Society of America, December 1, 1945, at Princeton, N. J.

⁷ C. L. Huggie, J., and Wishart, F. O., *Brit J Exp Path*, 1934, **15**, 390.

⁸ Beard, J. W., Finkelstein, H., and Wyckoff, R. W. G., *J Immunol*, 1938, **35**, 415.

⁹ Beard, J. W., Bryan, W. R., and Wyckoff, R. W. G., *J Infect Dis*, 1939, **65**, 43.

¹⁰ Taylor, A. R., *J Biol Chem*, 1946, **163**, 283.

⁶ We are greatly indebted to Dr. Max Delbruck, Vanderbilt University, for the strain of bacteriophage and its host and for advice in the problems of cultivation.

¹¹ Anderson, T. F., *J Cell and Comp Physiol*, 1945, **25**, 17.

¹² Hook, A. E., Taylor, A. R., Sharp, D. G., and Beard, J. W., to be published.



Fig 2

Photograph of sphere (tennis ball) and the shadow cast by light striking at the angle tangent $2/7$

in which W = weight of metal evaporated in grams r = distance (7 cm) from the metal to the virus preparation and ρ = the density of the metal (19 for gold, 7 for chromium) The thickness of the deposit on the film lying at an angle ϕ to the beam was less by the factor $\tan \phi$ which in this case was $2/7$ or $1/5$ Under the conditions of the experiments the thickness of the layer of gold on the normal surface was about 8.5 \AA per mg of metal evaporated the values for chromium were greater by the quotient of the densities, or 2.7 times as much

For purposes of comparison, a photograph was made of a spherical object a tennis ball, and the shadow produced by light striking it at the angle of $\tan 2/7$

Results The rabbit papilloma virus in conventional electron micrographs¹³ is seen as circular or rounded images of low contrast which merge indistinctly into the background at the periphery On the basis of apparent uniformity in the size and shape of the images, the virus was judged to be essentially spherical in shape In Fig 1 there is shown the appearance of the virus shadowed with 10 mg of gold Most of the images are highly uniform in shape and size others, which are irregular in size and shape, have the appearance of representing fragments of virus par-

ticles

The characters of the shadow cast by a sphere a tennis ball, lighted at an angle of tangent $2/7$, are shown in Fig 2 On comparison of Fig 1 with Fig 2, it is seen that the shadows of the virus particles differ greatly from the shadow cast by the ball The shadows of the virus are nearly triangular in shape, relatively very broad at the base and the length is, on the average, about $2/3$ of what it would be if the height of the particles were equal to the diameter of the images Judging from these characters it is clear that the particles, at the moment of shadowing, were not spherical but were flattened on the under and possibly on the upper sides as well

Vaccinia virus deposited on the collodion membrane from 0.005 M phosphate buffer and washed by dipping in water is pictured in Fig 3 As described by Green, Anderson and Smadel,¹⁴ the images are approximately rectangular in shape and give evidence of differentiation in the internal structure of the particle as shown by the presence of rounded regions of density greater than that of the remainder of the particle The particles lightly washed seem to be coated with a sticky material which causes coherence of some of the particles and in which bubble-like structures appear

Vaccinia elementary bodies shadowed with 8 mg of gold are seen in Fig 4 In contour in the horizontal plane the images of Fig 4 are similar to those of Fig 3 except for roughness and unevenness about the edges, which may be related either to uneven deposit of gold or possibly to coating of the bubble-like structures seen in Fig 3. The bodies are clearly flattened in varying degree, as shown both by contour and length of the shadows The shadows are not sharp in the region of the apex where the metal shades far into the shadow One of the images seen in Fig 4 is almost circular and of very high contrast The shadow associated with it is much longer than the shadows of the other particles This appearance suggests, as discussed below that the image represents

¹³ Sharp, D G Taylor, A R, Beard D and Beard, J W Proc Soc Exp Biol and Med 1942 50 205

¹⁴ Green, R H, Anderson T F, and Smadel, J E J Exp Med, 1942, 75, 651

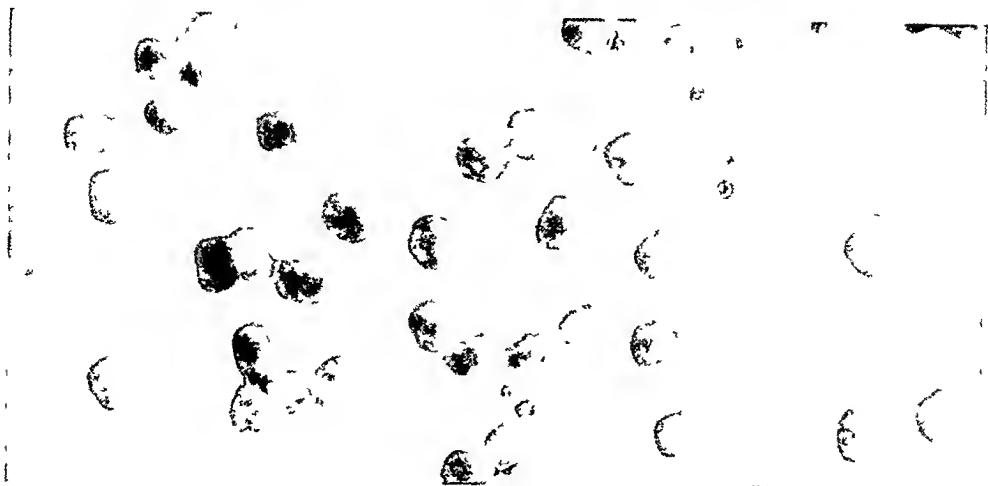


Fig 3

Conventional electron micrograph of vaccinia elementary bodies Magnification 25,000 \times

one of the particles standing on end. In many of the images there are seen relatively broad and low mound-like protrusions that may represent the effects of internal structure, possibly the material of the regions of high density of Fig 3, extending up beneath the surface.

The T_2 bacteriophage of *E. coli* is shown in the electron micrograph of Fig 5. The purified bacteriophage was suspended in physiological saline solution, and the concentrate was diluted $\frac{1}{5}$ with 0.023 M CaCl_2 for preparation of the film. Similar to the findings of Luria and Anderson,¹⁷ the images of the bacteriophage are tadpole-shaped, showing the presence of a large head and a stubby tail tipped with a ball- or disc-shaped knob. The heads, which are of high capacity to absorb electrons, are uniform in size and hexagonal in shape. The tails, which show low electron-stopping power, are uniform in length but appear to vary in width. No internal structure was visible in the presence of the calcium salt.

In Fig 6 are shown bacteriophage particles shadowed by the evaporation of 3.7 mg of chromium. The heads do not give the shadows either of ideal spheres, or even of regular polyhedrons, but rather those of short,

somewhat flattened rods with conical caps. The stubby tail, of uneven thickness terminates in a ball- or disc-shaped structure. The tail shadows are likewise short, indicating a flattened condition consistent with the observed low electron-stopping power. The degree of flattening is not the same in all preparations nor has it been the same for the individuals of a given picture, as may be seen in Fig 6.

Discussion The application of the shadow technic adds greatly to the results of direct observation of viruses in the electron microscope. Coating of the virus preparation is effected *in vacuo* under conditions not greatly different from those to which the preparation is subjected in the course of conventional electron micrography. Thus the characters portrayed with 3-dimensional effect in the shadow pictures are those of the dried and possibly shrunken and distorted virus particles and are probably not quantitatively representative of the particles in their native state in aqueous suspension. Measurements of shadows¹ of tobacco mosaic virus rods yield values of rod height similar to results obtained for rod width by means of X-ray examination¹⁶ and measurements in electron micro-

¹⁶ Beinl, J. D., and Fankuchen, I., *J. Gen. Physiol.*, 1941, 25, 111.

¹⁷ Luria, S. E., and Anderson, T. F., *Proc. Nat. Acad. Sci.*, 1942, 28, 127.

¹⁸ Stanley, W. M., and Anderson, T. F., *J. Biol. Chem.*, 1941, 139, 325.

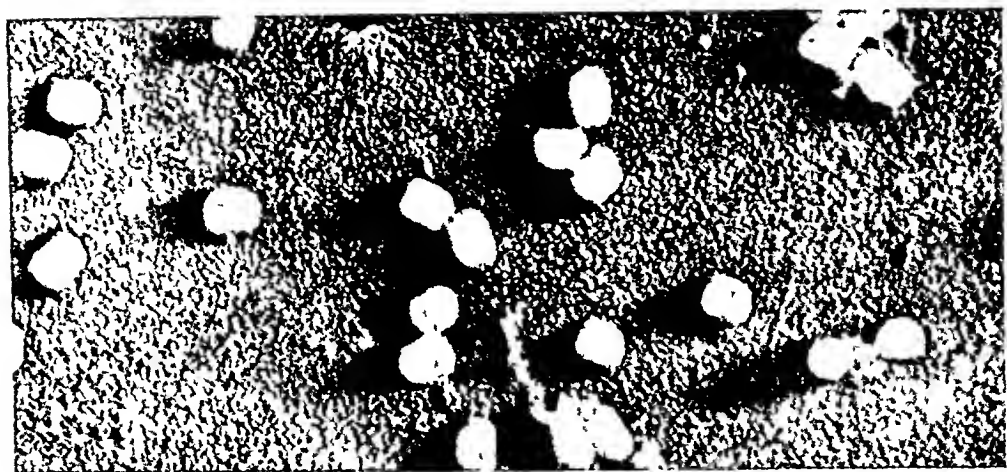


Fig. 4

Vaccinia virus shadowed with 8 mg of gold at the angle tangent $2/7$ Magnification 25,000 \times



Fig. 5

Conventional electron micrograph of the T_2 bacteriophage of *E. coli* Magnification 42,700 \times

graphs¹⁷ That shrinkage of other viruses occurs under these conditions, however is indicated by the observation of particle diameter in electron micrographs smaller than values obtained in studies of the virus in aqueous suspension¹⁸

Shadow electron micrographs of the papilloma virus corroborate evidence of uniformity of particle size and shape obtained in sedimentation studies¹⁹ and from conventional

electron micrographs¹³ In the shadow micrography the apparent height of the particles is about $\frac{2}{3}$ that expected of spheres a value much greater than that for oblate spheroids of axial ratio 11-1 previously predicted¹⁹ from sedimentation, diffusion and viscosity data on the assumption that the particle is unhydrated Recent studies²⁰ of

¹⁸ Sharp D G, Taylor, A R, McLern I W, Jr, Beard, D and Beard, J W, *J Biol Chem*, 1945, 159, 29

¹⁹ Neurath, H, Cooper G R, Sharp, D G, Taylor, A R, Beard D and Beard J W, *J Biol Chem*, 1941 140, 293

²⁰ Sharp, D G, Taylor A R, and Beard, J W, *J Biol Chem*, 1946, 163, 289

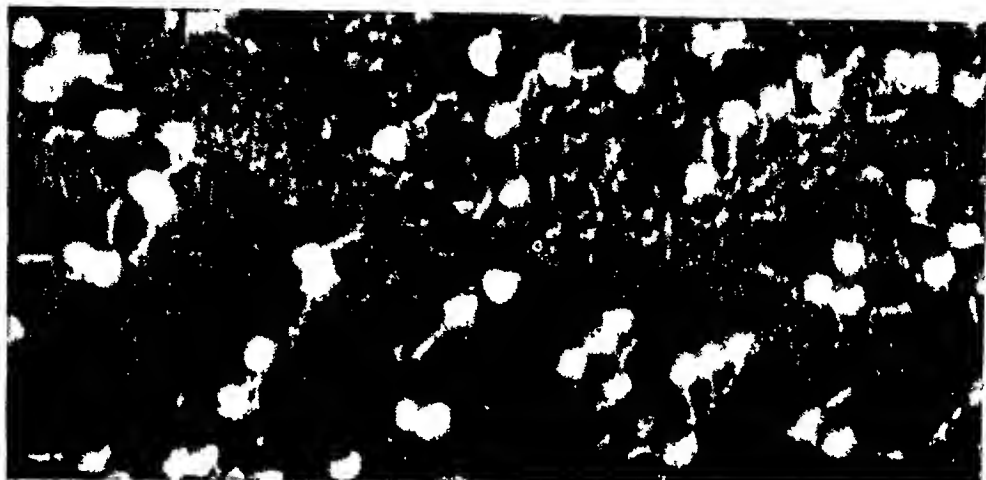


Fig 6

Baetomophage shadowed with 3.7 mg of chromium at the angle tangent $1/5$ Magnification 42,700 \times

the density of the papilloma virus in aqueous suspension indicate that the particles are 58% water (by volume) and, if spherical in shape, about 66μ in diameter. The difference between this value and 44μ observed in electron micrographs¹³ is probably, in part, a measure of the shrinkage occurring in the plane of the picture. Greater shortening of the vertical diameter than of that in the horizontal plane is not improbable, since settling and spreading of the wet particle on the collodion membrane might be expected. This is consistent with the obvious flattening of the particles on the under side indicated by the shadows broad at the base. Quantitatively, the shadow pictures show that the dry papilloma virus particles are slightly flattened spheroids, the finding is not incompatible with the view that the virus is essentially spherical in the wet state and flattens on drying in contact with the collodion membrane.

The elementary bodies of vaccinia have been described as "brick-shaped"¹⁴ which implies a difference between the height and the width of the particles lying on the collodion membrane. Judging from the contour in the shadow pictures, the dry bodies seem greatly flattened, but this appearance is not wholly consistent with the length of the shadows. This discrepancy and the lack of sharpness of the shadows may be explained, at least

in part, on the assumption that shrinkage of the particle continues during the process of metal-coating. The particles, even after drying, are clearly well-rounded about all diameters. There is some evidence that the particles are not brick-shaped but are essentially short cylinders or rods. In a large number of micrographs, the width or short dimension of particles has been relatively uniform, and no evidence has been seen of bodies lying on edge. On the contrary, circular images of high contrast and of diameters similar to the short dimension of the rectangular images occur with a frequency of about 1 or 2 per hundred. A shadow image of this sort is shown in Fig 4. The circular shape, and the relatively high contrast of the image, together with the greater length of the shadow, may be interpreted as evidence that this particle is a short rod or cylinder standing on end. The consistency of the findings suggests that the body casting this image differs from the others only in orientation, and that the vaccinia virus may be essentially rod-shaped.

The mound-shaped protrusions seen in the shadow images are interpreted as representing the intraparticular material of high contrast in conventional electron micrographs. The structure appears relatively large and of greater density and resistance to shrinkage than the remainder of the particle.

The highly uniform width, the well-rounded contours and the length of the shadows are characters suggesting that the headpieces of the bacteriophage are likewise probably short cylinders. The hexagonal shape in ordinary electron micrographs indicates a conical shape of the ends of the rods. In the absence of information of the density, size and water content of the bacteriophage in aqueous suspension no estimate can be made of the possible degree of shrinkage on drying.

Summary Electron micrographs of the rabbit papilloma and vaccinia viruses and the

T₂ bacteriophage of *E. coli* were obtained with the metal-shadowing technic. The papilloma virus dried *in vacuo* for application of the metal appeared spheroidal in shape, flattened especially at the region of contact with the film. The vaccinia virus was greatly flattened and showed the presence of a denser internal material bulging beneath the surface. The bacteriophage was a tadpole-shaped structure with a headpiece of well-rounded contours and a stubby tailpiece. The findings were discussed with respect to their bearing on the shape of the viruses in the wet state.

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Attempts to Propagate Murine Poliomyelitis Virus on Various Intestinal Bacteria and Protozoa *

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Studies of microbial metabolism have amply demonstrated that many bacteria and protozoa are endowed with a complex equipment of enzymes that are utilized for cellular propagation. It is therefore not surprising that numerous attempts have been made to grow certain pathogenic viruses in symbiosis with bacteria or other free-living cells.¹ Without passing on the merits of these observations, the relationship between the virus of poliomyelitis and the intestinal flora seemed to us of particular interest in this connection. As is well known, human virus—with suf-

ficient neurotropic virulence to permit intracerebral transfer to monkeys—can often be isolated from the faeces of cases or healthy contacts. More important yet, normal albino mice harbor regularly in the intestinal tract neurotropic murine virus (Theiler virus) which apparently persists for the lifetime of the animal. Additional evidence of the remarkable affinity of poliomyelitis virus for the alimentary canal is furnished by experience with the mouse-adapted strain of human SK poliomyelitis virus which could be recovered from the faeces of 3 different infected hosts, natural or experimental, i.e., man,² monkey³ and guinea pig.⁴ How a supposedly strictly neurotropic virus can maintain itself for any protracted period in the absence of living nervous material is dif-

* Aided by grants from the Philip Hanson Hiss, Jr., Memorial Fund, the Warner Institute for Therapeutic Research, and anonymous donors.

¹ a Nicolau, S., and Lwoff, A., *Bull. Soc. Path. Ex.*, 1932, **25**, 721; b Silber, L. A., and Wostrachow, E. I., *Centralbl. Bakt. Orig.*, 1933, **129**, 389; 1933, **129**, 396; 1934, **132**, 314; c Silber, L. A., and Dosser, E. M., *Centralbl. Bakt. Orig.*, 1933, **131**, 222; d Silber, L. A., and Timakow, W. D., *Centralbl. Bakt. Orig.*, 1935, **133**, 242; e Poppe, K., and Busche, G., *Centralbl. Bakt. Orig.*, 1936, **136**, 385.

² Trask, J. D., Vignee, A. J., and Paul, J. R., *Proc. Soc. Exp. Biol. and Med.*, 1938, **88**, 147; *J. Am. Med. Assn.*, 1938, **111**, 6.

³ Trask, J. D., and Paul, J. R., *Ann. Int. Med.*, 1942, **17**, 975.

⁴ Ibanez, J. S., *Trabajos del Instituto Cajal de Investigaciones Biologicas*, 1944, **36**, 137.

difficult to understand. The theory could therefore be advanced that the infectious agent may possess a vicarious enterotropism which permits viral propagation, at a saprophytic level, on enteric microorganisms normally present in the intestinal canal.

To test the validity of this hypothesis experiments were undertaken in which cultures of certain intestinal bacteria or protozoa (including some non-intestinal parasites)[†] were examined for their ability to serve as a substrate for *in vitro* propagation of murine poliomyelitis virus. A mouse-adapted strain of human poliomyelitis virus (MM) which grows well in embryonic mouse and fairly well in chick tissue cell culture was used in this work.⁵ This virus was chosen because its extremely high virulence for mice by both intracerebral and intraperitoneal routes facilitated the detection of minimal amounts of virus; moreover, its marked resistance against physical and chemical agencies offered some assurance for survival of the virus under unfavorable environmental conditions. The microorganisms employed as test substrates for possible viral propagation were the following: (1) Mixed bacterial flora obtained from human faeces, (2) *B. coli* communis, (3) *Leptospira biflexa*, (4) *Entamoeba coli*, (5) *Trichomonas hominis*, (6) *Chilomastix mesneli*, (7) *Tetrahymena gelii*, (8) *Leishmania donovani*, (9) *Trypanosoma gambiense*. The general experimental procedure consisted of combining sufficient 10% viral mouse brain suspension with the different bacterial or protozoal cultures in their

respective optimal media so as to yield a final 1:100 virus concentration. After proper incubation such mixtures were transferred to new culture media in amounts constituting a further 10-fold dilution of virus with serial passages being maintained for several subsequent generations. For control purposes, 10% viral brain suspension, in identical proportions, was added to the respective sterile culture media which, after similar incubation, were transferred serially to new culture media, thus yielding progressively increasing 10-fold dilutions of virus alone. The 2 sets of tubes, *i.e.*, microbial cultures and controls, were examined at each passage for the presence of viable organisms by microscopic count and for the presence of virus by the injection of albino mice. In the case of the enteric bacteria it was necessary to use Seitz filtrates for injection, whereas the protozoal culture and control tubes could be injected safely without previous filtration. All injections were carried out intracerebrally with 0.03 cc of test fluid, except for cultures of *Entamoeba coli*, *Chilomastix mesneli* and *Trichomonas hominis* which on account of their concomitant bacterial flora were tested by intraperitoneal injection of 0.1 cc amounts. The injected mice were carefully observed for the development of characteristic symptoms. Tissues of animals which died without definite signs of paralysis were further checked by subpassage to new mice for final identification of the virus. The results are shown in Table I.

It will be seen from Table I that none of the tested bacteria or protozoa of increasingly differentiated structure were capable of supporting neurotropic MM virus beyond the number of passages which maintained virus in control tubes, with the possible exception of the *Trichomonas hominis* culture which was therefore studied in more detail. Thus, active virus was clearly demonstrable in the protozoal-bacterial culture up to and including the fourth serial passage, whereas virus alone in the uninoculated medium failed to survive beyond the initial passage in repeated tests. The impression was first gained that virus had actually multiplied to some ex-

[†] Grateful acknowledgement is made of the receipt of the protozoal cultures from the following sources: *Entamoeba coli*, *Trichomonas hominis* and *Chilomastix mesneli* from Dr. D. Guisti, Dept. of Preventive Medicine, College of Medicine, New York University; *Tetrahymena gelii* from Dr. Kessler, College of Physicians and Surgeons, Columbia University; *Leishmania donovani* from the National Institute of Health, Washington, D.C.; *Trypanosoma gambiense* from Dr. D. Weiman, Department of Comparative Pathology and Tropical Medicine, Harvard University Medical School.

⁵ Jungeblut, C. W., and Dalldorf, G., *Am. J. Publ. Health*, 1943, 33, 169; Jungeblut, C. W., *J. Exp. Med.*, 1945, 81, 275.

difficult to understand. The theory could therefore be advanced that the infectious agent may possess a vicarious enterotropism which permits viral propagation, at a saprophytic level, on enteric microorganisms normally present in the intestinal canal.

To test the validity of this hypothesis experiments were undertaken in which cultures of certain intestinal bacteria or protozoa (including some non-intestinal parasites)[†] were examined for their ability to serve as a substrate for *in vitro* propagation of murine poliomyelitis virus. A mouse-adapted strain of human poliomyelitis virus (MM) which grows well in embryonic mouse and fairly well in chick tissue cell culture was used in this work.⁵ This virus was chosen because its extremely high virulence for mice by both intracerebral and intraperitoneal routes facilitated the detection of minimal amounts of virus, moreover, its marked resistance against physical and chemical agencies offered some assurance for survival of the virus under unfavorable environmental conditions. The microorganisms employed as test substrates for possible viral propagation were the following: (1) Mixed bacterial flora obtained from human faeces, (2) *B. coli* communis, (3) *Leptospira biflexa*, (4) *Entamoeba coli*, (5) *Trichomonas hominis*, (6) *Chilomastix mesneli*, (7) *Tetrahymena geleii*, (8) *Leishmania donovani*, (9) *Trypanosoma gambiense*. The general experimental procedure consisted of combining sufficient 10% viral mouse brain suspension with the different bacterial or protozoal cultures in their

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⁵ Jungeblut, C. W., and Dalldorf, G., *Am. J. Publ. Health*, 1943, **33**, 169; Jungeblut, C. W., *J. Exp. Med.*, 1945, **81**, 275.

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and that slightly higher concentrations were lethal¹⁵ In view of these facts it became of interest to study the action of thiourea upon the development of the sea urchin egg whose normal embryology is well known

Material and Methods The material used in this study consisted of eggs of ripe female sea urchins obtained during the months of July and August All of the available eggs from each sea urchin were placed in a container and fertilized The fertilized eggs were transferred to 'finger bowls' containing 200 cc of test solution which was maintained at 21-22.5°C Each experiment was confined to one egg batch, and several hundred eggs were placed in each of the test solutions The observations which are to be described below are the results of 25 experiments carried out under the above conditions Test solutions consisted of 0.1% to 1% of thiourea in sea water Similar concentrations of urea in sea water, and untreated sea water served as controls

Cleavage rates and development were ascertained by examining samples of the cultures taken at suitable intervals for periods ranging from 3 to 4 hours for early cleavage and for as long as 72 hours for subsequent development For the purpose of examining the structure of some of the embryos in greater detail, specimens were fixed, sectioned and stained in the usual manner

Cleavage and Development When fertilized sea urchin eggs were exposed to concentrations of 1% thiourea, the eggs as a rule failed to undergo cleavage In similar concentrations of urea, on the other hand, cleavage proceeded at a relatively normal rate

In contrast to the effect noted above, when the eggs were exposed to a 0.5% solution of thiourea early cleavage occurred at a normal rate until the blastula stage was reached Average results showed that 24 hours after fertilization, the embryos were still in the blastula stage, at 48 hours, the archenteron had usually appeared, but even as long as 72 hours after fertilization, the coelom had not as yet differentiated In the course of normal development gastrulae develop in about 12 hours and after 24 hours plutei have already differentiated

Examination of the embryos which had been exposed to 0.5% solutions of thiourea showed that gastrulation had been almost completely inhibited While the untreated gastrulae attain a size of about 70 μ and then differentiate into plutei, the treated specimens ceased differentiating at the incomplete gastrula stage, but continued to increase in size until they attained a diameter of 90 μ The ectoderm in these specimens appeared normal, but the primitive mesoderm consisted of a few large cells arranged in irregular patches Occasionally the first skeletal element, the triradiate spicules, differentiated, but pigmentation of the specimens was as a rule abnormal

Exposure to concentrations of thiourea ranging from 0.1 to 0.3% did not appreciably affect the cleavage rate When the eggs were allowed to develop for as long as 72 hours, however, it was observed that there was a progressive decrease in the rate of development of the plutei which was directly proportional to the concentration of the drug

Inhibition and Recovery After repeated observations had established the fact that fertilized sea urchin eggs failed to undergo gastrulation in concentrations of 0.5% thiourea, a number of experiments were carried out at this concentration to ascertain more precisely the mechanism responsible for inhibition of gastrulation Accordingly several egg batches were fertilized and allowed to develop in sea water through the first, second and subsequent cleavages as far as the blastula and gastrula stage At appropriate intervals several hundred specimens at each of these stages were then placed in a solution of 0.5% thiourea and the subsequent development was observed In those individuals which had been transferred to the test solution prior to blastulation development proceeded normally until this stage was attained Gastrulation, however, failed to take place When swimming blastulae were transferred to the thiourea solutions gastrulation did not occur Introduction of well developed gastrulae into thiourea solutions, on the other hand, resulted in the development of plutei at a retarded rate

Analysis of the data obtained for the ex-

tent However, further experiments in which virus was added to a heat-killed *Trichomonas* culture showed that active virus could likewise be carried through 3 serial passages. It would therefore appear that no viral propagation had occurred in these tests and that the presence of the protozoan with its bacterial flora had merely served to render the unfavorable medium more favorable for survival of the originally inoculated virus.

Conclusions Propagation of human or simian strains of poliomyelitis virus in any kind of culture medium is extremely difficult whereas certain murine strains will grow well in tissue culture containing embryonic mouse

brain. A number of enteric bacteria and protozoa as well as some highly differentiated non-intestinal protozoa were examined for their ability to permit propagation of MM murine poliomyelitis virus in culture. No evidence of viral propagation was found as determined by mouse inoculation. The data do not encourage the assumption that neurotropic poliomyelitis virus in stool or sewage may be maintained in symbiosis with free living microbial cells. However the limited extent of this work does not exclude the possibility that experiments with other marine or faecal organisms under different experimental conditions might be more successful.

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Effect of Thiourea on Development of the Sea Urchin *Ambacia Punctulata*

GERRIT BEVELANDER * (Introduced by E. D. Goldsmith)

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Introduction The development of the rat,¹ the frog,⁴⁻⁶ and the fish⁷ has been influenced by treatment with thiourea. These developmental changes have been attributed to a state of functional hypothyroidism resulting from an interference of thiourea with

the production of normal thyroid hormone.⁸⁻¹⁰ It has been suggested that the drug may act by inhibiting the peroxidase¹¹ or the cytochrome oxidase¹² activity of the thyroid gland. From additional studies,¹³ however, it appears that thiourea inhibits an oxidative enzyme other than cytochrome oxidase. Recently it has been shown, that low dosages of thiourea incorporated in the food medium produced defects in developing *Drosophila*,¹⁴

* With the technical assistance of Miss Myrna Helfman

¹ Hughes, A. M., *Endocrinology*, 1944, **34**, 69

² Goldsmith, E. D., Gordon, A. S., and Charipper, H. A., *Federation Proc.*, 1944, **3**, 13

³ Goldsmith, E. D., Gordon, A. S., and Charipper, H. A., *Am. J. Obstet. and Gynecol.*, 1945, **49**, 197

⁴ Gordon, A. S., Goldsmith, E. D., and Charipper, H. A., *Nature*, 1943, **152**, 504

⁵ Hughes, A. M., and Astwood, E. B., *Endocrinology*, 1944, **34**, 138

⁶ Gordon, A. S., Goldsmith, E. D., and Charipper, H. A., *Growth*, 1945, **9**, 19

⁷ Goldsmith, E. D., Nigrelli, R. F., Gordon, A. S., Charipper, H. A., and Gordon, M., *Endocrinology*, 1944, **35**, 132

⁸ Keston, A. S., Goldsmith, E. D., Gordon, A. S., and Charipper, H. A., *J. Biol. Chem.*, 1944, **152**, 241

⁹ Franklin, A. L., Lerner, S. R., and Chirikoff, I. L., *Endocrinology*, 1944, **34**, 265

¹⁰ Larson, R. A., Keating, F. R. Jr., Peacock, W., and Rawson, R. W., *Endocrinology*, 1945, **36**, 160

¹¹ Dempsey, E. W., and Astwood, E. B., *Endocrinology*, 1944, **34**, 27

¹² Paschalis, K. E., Cantarow, A., and Tillson, E. K., *Proc. Soc. Exp. Biol. and Med.*, 1945, **60**, 148

¹³ Lerner, S. R., and Chirikoff, I. L., *Endocrinology*, 1945, **37**, 368

¹⁴ Hurnly, M. H., and Goldsmith, E. D., in preparation

¹⁵ Goldsmith, E. D., and Hurnly, M. H., *Science*, in press

Effect of Elevated Body Temperature on Plasma Vitamin A and Carotene *

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AND GEORGE X SCHWEMLEIN

(With the technical assistance of Elsie Miller and Helen Wharton)

From the Chicago Intensive Treatment Center, Chicago Board of Health, in cooperation with the
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Elevation of the body temperature is well recognized as a therapeutic procedure "Fever-therapy" is the term now applied to systemic heating of the human body¹ It has been shown that the therapeutic efficacy of mapharsen, nearsphenamine, penicillin² and the sulfonamides is increased during physically-induced fever The addition of bismuth enhances the clinical results in the intensive arsenotherapy of early syphilis, although the mode of action has not been well understood³ Fever, in combination with these agents (fever-chemotherapy), is a method of intensive treatment applied to syphilis and gonorrhea⁴

In fever-chemotherapy there are 3 known variables the height to which the body temperature is elevated, the number of hours this temperature is maintained,⁵ and the amount and type of chemotherapy administered

A number of biochemical studies on body fluids have been made under the influence of physically-induced fever⁶ To our knowledge, no biochemical determination has been found which might serve as an indicator of the intensity of the action of fever-chemotherapy on the human organism It was believed that a systematic study of the plasma vitamin A level and that of its precursor, carotene, might furnish valuable information concerning the influence of the elevation of body temperature, and serve as a guide in comparing the effects of various combinations of fever-chemotherapy

A depression of the plasma and serum vitamin A has been found during febrile infectious diseases, especially pneumonia⁷ This

* The authors are greatly indebted to Dr Herman N Bundesen, President, Chicago Board of Health who made this investigation possible and took an inspiring interest in the work Dr Theodore J Bruer, Senior Surgeon, U S P H S, Venereal Disease Control Officer, cooperated in the organization of this study

¹ Krusen, F H, *Physical Medicine*, Philadelphia and London, 1941 45

² Bork, R A, Carpenter, C M, and Warren, S L, *Am J Syph, Gonorr and Ven Dis*, 1942, 26, 282, Stokinger, H E, Dorn, F L, Bork R A, and Carpenter, C M, *Am J Syph, Gonorr and Ven Dis* 1944, 28, 465, Bork, R A, Dorn, F L, and Carpenter, C M *Am J Syph, Gonorr and Ven Dis*, 1945, 29 428, Moore, J E, *Am J Syph, Gonorr and Ven Dis* 1945, 29, 195, Engle, H, and Munschman, A I, *J Exp Med*, 1944, 80, 493

³ Engle, H, and Hogan, R B, *Science*, 1942, 95, 360, Stokes, J H, Beerman, H, and Warrnack, H, *Am J M Sc*, 1943, 206, 621, Rattner, H, *J A M A*, 1943, 122, 986

⁴ Bundesen, H N, Bruer, T J, and Kendall, H W, *J A M A*, 1943, 123, 816, Simpson W M, Kendall, H W, and Rose, D L, *Suppl No 16 to Ven Dis Inform*, 1942, Rose, D L,

Kendall, H W, and Simpson, W M, *War Med*, 1941, 1, 470, Thomas, E W, *N Y State J. Med*, 1944 44, 157, Jones N, Carpenter, C M, Bork, R A, Warren, S L, and Hanson, H, *Ven Dis Inform* 1944, 25, 99

⁵ Bork, R A, Carpenter, C M, and Warren, S L, *J Exp Med*, 1932, 56, 741, 751

⁶ Krusen F H *Physical Medicine*, Philadelphia and London, 1941, 65, Osborne, S L, and Holmquist, H J, *Technic of Electrotherapy and Its Physical and Physiological Basis*, Springfield and Baltimore 1944, 682

⁷ Krusen, S W, and McCoord, A B, *J Ped*, 1938, 13, 635, Thiele, W, and Scheiff, J J, *Klin Wchenschn*, 1939, 18, 1275, May, C D, Blehman, K D, McCreary, J F, and Allen, F H, *Am J Dis Child*, 1940, 59, 1167, Josephs, H W, *Am J Dis Child*, 1943, 65, 712

periments described above shows that inhibition of gastrulation does not have its inception in the early cleavage stages and that regardless of previous treatment, thiourea acts selectively upon the blastula to prevent gastrulation, and finally, once gastrulae are differentiated, development as a whole is retarded in 0.5% thiourea, but does nevertheless take place.

In order to ascertain whether the inhibitory effects produced by exposure to thiourea were reversible, several batches of eggs were exposed to concentrations of thiourea ranging from 0.1% to 1% for periods ranging from one hour to 72 hours. They were then washed and returned to sea water. In general, the higher the concentration and the longer the exposure to the drug, the less the recovery, and conversely, with short exposure periods and low concentrations, from 50-90% of the specimens developed into normal plutei after they had been returned to sea water.

Discussion The observations on the development of thiourea treated sea urchin eggs disclose that exposure to the higher concentrations, 1% or greater, completely inhibit cleavage. Lower concentrations, 0.5%, on the other hand, specifically inhibit gastrulation, while the lowest concentrations found to be effective, 0.1% to 0.3%, produced a retardation in the overall growth rate of the plutei. In the higher concentrations, the inhibitory effects are irreversible, in the intermediate range, reversibility of inhibition takes place to a limited degree, while in the lowest concentrations that were tested, the inhibitory

effects are completely reversible.

It is thus apparent that the various physiological processes which operate during cleavage, gastrulation, and growth have different thresholds of inhibition in respect to exposure to thiourea.

Since development is dependent upon the function of a number of enzyme systems and since, further, it has been reported^{12, 13} that thiourea inhibits certain enzymes which are present in a developing sea urchin egg, it is tentatively suggested that the mechanism by means of which development is inhibited by thiourea in both a general and a selective manner may be most readily accounted for by assuming that thiourea inhibits enzyme systems which are necessary for the differentiation and growth of the sea urchin.

Summary Sea urchin eggs which were subjected to varying concentrations of thiourea showed a number of pronounced effects. Concentrations of 1% or more resulted in a complete inhibition of cleavage. In 0.5% solutions of thiourea, the eggs developed normally until the blastula stage was reached. For as long as 72 hours, the eggs did not differentiate beyond the gastrula stage. When the arrested gastrulae were returned to sea water, the inhibitory effects were reversible.

In the lowest concentrations used, which ranged from 0.1% to 0.3%, early cleavage was not affected. There was, however, a retardation in the rate at which the plutei developed, which was observed to be proportional to the concentration of the drug.

TABLE II

Maximum and Minimum Values for Plasma Vitamin A, Carotene Total Plasma Protein and Blood Hemoglobin per 100 cc of 92 Individual Patients before Elevation of Body Temperature, Compared with Values Formerly Obtained and Published by Other Authors

Patients studied	Vitamin A, I U		Carotene μ g		Total protein, g		Hemoglobin, g	
	Max	Min	Max	Min	Max	Min	Max	Min
92 (this study)	170	36	163	22	8.25	6.25	17.0	8.6
434 (formerly studied) ¹¹	189	25	282	28				
(values of other authors quoted from 11)	300	28	347	18				

hours of fever-chemotherapy at 105.0°F (rectal), the plasma vitamin A levels were found to be in about the same range as before treatment in 9 patients.

The patients receiving 3 and 6 hours of fever-chemotherapy at 106.0°F (rectal) or 8 hours at 105.5° to 106.0°F (rectal) showed a decline of the plasma vitamin A and carotene which lasted until the first morning after treatment, except in the 6-hour fever and penicillin group, where a small rise occurred on the first morning after fever. On the second morning after fever a marked rise of the plasma vitamin A was recorded and on the third morning after therapy, the plasma vitamin A had returned approximately to the prefever level.

In 3 groups, the medians and the means for vitamin A after fever reached higher values than were recorded before fever. This is similar to the recently reported hypervitaminemia A¹⁴ observed following recovery from infectious febrile diseases.

The changes in the plasma vitamin A were most pronounced in those patients receiving fever-chemotherapy of 8-hour duration. The course of the plasma vitamin A recorded in I U per 100 cc plasma for each individual patient in this group is demonstrated in Fig. 1. There are some variations in the extent of these changes but the pattern is practically the same, i.e., a marked decline after fever-chemotherapy, followed by a rapid spontaneous return toward the prefever level.

For each of the 7 groups of patients who were given different combinations of fever and chemotherapy the median values and the arithmetic means of plasma vitamin A, carotene, total protein and hemoglobin were calculated. In almost all instances the median values and the arithmetic means were similar. A comparison of these values indicated that the course of the plasma vitamin A and carotene is definitely influenced by the number of hours the body temperature is maintained at 105.0° or 106.0°F (rectal), irrespective of the type of chemotherapeutic drugs given in combination with fever. Therefore, the patients were divided into 3 groups, according to the number of hours the body temperature was elevated to 105.5° or 106.0°F (rectal), to analyze the effect of the duration of fever on plasma vitamin A and carotene.

Group I—Patients receiving 8-hour fever at 105.5° to 106.0°F (rectal).

Group II—Patients receiving 6-hour fever at 106.0°F (rectal).

Group III—Patients receiving 3-hour fever at 106.0°F (rectal).

The median values and the arithmetic means of vitamin A, carotene, total protein and hemoglobin for each of these 3 groups were compiled. Again, both medians and arithmetic means are practically identical. The results for vitamin A, carotene and total protein are demonstrated graphically in Fig. 2.

The results of these studies show that the plasma vitamin A is depressed when the body temperature is elevated to 105.5° or 106.0°F.

¹⁴ Steigmann, F., Meyer, K. A., and Popper, H., *Ann. Int. Med.*, 1945, 22, 832.

TABLE I
Types of Fever Chemotherapy Studied

No patients studied	Clinical diagnosis			Chemotherapeutic drugs administered		°F temp level	Hr main tained fever
	Primary	Secondary	Early latent	Before fever	During fever		
10	2	6	2	150 mg elemental bismuth (bismuth subsalicylate in oil) I M	176 mg mapharsen per kg body wt	105.5	8
26	1	14	11	same	same	106	6
21	8	13	0	600,000 O U penicillin I M	600,000 O U penicillin I M	106	6
8	2	6	0	same	600,000 O U penicillin I V	106	3
9	CNS syphilis			none	60 mg mapharsen	105	3
12	Sulfonamide resistant gonorrhea			8 g sulfathiazole	none	106	8
6	4	"	"	sulfathiazole, penicillin or none	"	106	6
	2 gonorrhea relapsing after penicillin						
92 total							

depression of the plasma vitamin A, in all probability, is produced by the retention of vitamin A in the liver.⁸ The results of recent investigations indicate that, in general, the regulation of vitamin A levels in the blood is controlled by the liver.⁹ Hepatitis, associated with rise of icterus index, may be encountered in intensive fever-chemotherapy.¹⁰

The purpose of this investigation was to study the influence of the elevation of the body temperature by physical means⁴ in combination with various chemotherapeutic drugs on plasma vitamin A and carotene. Blood hemoglobin and total plasma protein determinations were also made to evaluate the possibility of hemo-concentration or dilution during the course of the study.

Experimental Procedures and Results
Studies were made of the effects of 7 different combinations of fever-chemotherapy upon plasma vitamin A and carotene in 92 patients, taken at random from April, 1943

⁸ Landquist, T, *Klin Wehnschr*, 1937, **16**, 1345, and *Act Med Scand Suppl No 97*, 1938

⁹ Balli, E P, Papper, E, Paley, K, and Baum, E, *Arch Int Med*, 1941, **68**, 102, Popper, H, and Steigmann, F, *J A M A*, 1943, **123**, 1108

¹⁰ Kendell, H W, Rose, D L, Miller, E, and Simpson, W M, *Arch Physical Med*, 1945, **26**, 76, Wallace, J, and Bushby, S R M, *Lancet*, 1944, **2**, 459, *Abst Ven Dis Inform*, 1945, **26**, 64

to March, 1945. These treatments and the clinical diagnoses are shown in Table I.

Vitamin A and carotene determinations were made according to Kimble's method with slight modification.¹¹ The Sheard Sanford Photometer¹² was employed for quantitative hemoglobin determinations. Specific gravity determinations were performed by the falling drop method of Kagan,¹³ from which plasma total protein values were calculated.

The initial values for vitamin A, carotene, total protein of the blood plasma and hemoglobin varied in individual patients over a considerable range (Table II).

A method was adopted to permit statistical and clinical comparisons of the results. The average (arithmetic mean) of 2 or 3 values for vitamin A, carotene, total protein and hemoglobin, found for each individual patient during the 5 days before fever-chemotherapy, was regarded as the base line and made equal to 100. All values found thereafter for the same patient were expressed in % of his prefever (base) value of 100.

On the first and second mornings after 3

¹¹ Abt, A F, Aron, H C S, Bimmerle, J F, Bundesen, H N, Delaney, M A, Fagen, H J, Farmer, C J, Wenger, O C, and White, J L, *Quart Bull Northwestern Univ Med School*, 1942, **16**, 241

¹² Sanford, A H, Sheard, C, and Osterberg, A E, *J Clin Path*, 1933, **3**, 405

¹³ Kagan, B M, *J Clin Invest*, 1938, **17**, 372

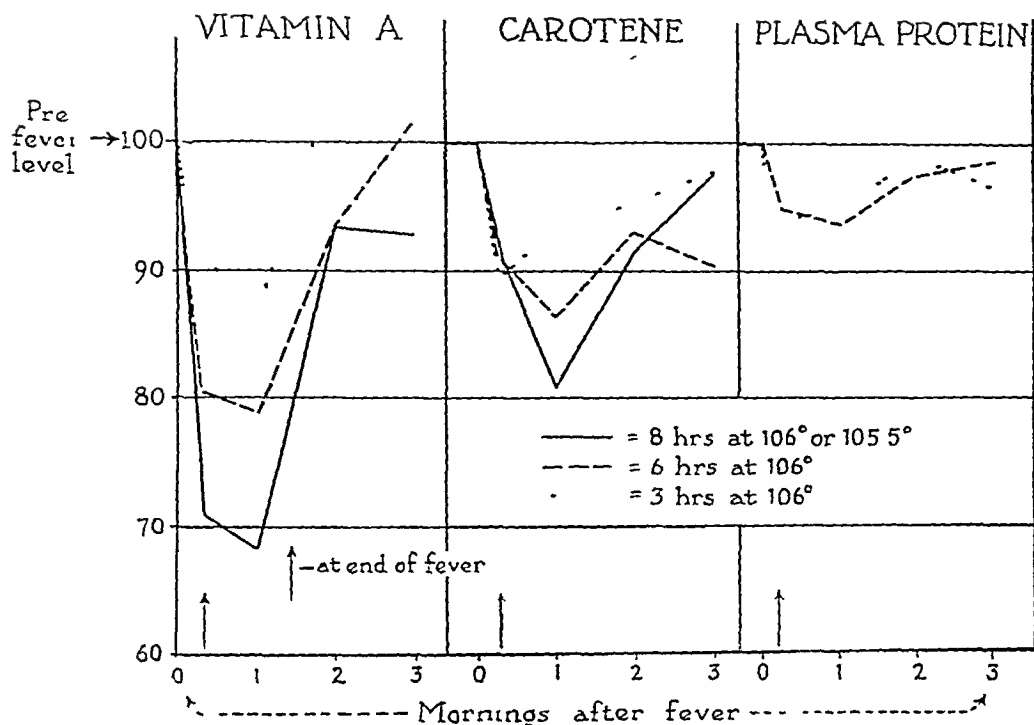


Fig 2

was present but not of sufficient magnitude to account for the decreases in the plasma vitamin A and carotene levels. This is particularly illustrated in the 8- and 6-hour fever-chemotherapy groups where the decrease in the levels was from 3 to 7 times that of the hemoglobin or total plasma protein. Fig 2 shows the changes in plasma protein. The changes in hemoglobin are parallel.

Each of the 22 patients given 8-hour sessions of fever-chemotherapy showed a depression of the plasma vitamin A of more than 15%. A total of 53 patients in the 3 groups were given 6-hour sessions of fever-chemotherapy at the level of 106.0°F (rectal), and all but one patient showed decreases of the plasma vitamin A level at the termination of the fever sessions or on the first morning after fever, as compared to the prefever levels. Five of these patients did have a depression of less than 10%, *i.e.*, within the possible range that might be attributed to the error of the method, for which no clinical explanation can be given. A depression of the plasma vitamin A level was found in all of

the 8 patients subjected to 3-hour fever-chemotherapy at 106.0°F (rectal). One of these patients had a depression of less than 10%. The number of hours the fever was maintained to some degree may account for this factor, as this phenomenon was not observed in the 8-hour fever sessions.

There was no significant difference in the vitamin A levels of those patients who experienced uneventful fever sessions as compared to those who had clinical evidence of dehydration, emesis or low blood pressure during or after fever-chemotherapy.

Four of the 84 patients who received fever-chemotherapy at the level of 105.5° to 106.0°F (rectal) developed mild jaundice with an icterus index[†] between 14 and 45 units. The plasma vitamin A values of 3 of these pa-

[†] Icterus index (II) was determined by comparison of the color of the serum against a standard solution of potassium dichromate in a block comparator.¹⁵

¹⁵ Simmons, J. H., and Gentzkow, C. J., *Laboratory Methods of the U. S. Army*, 5th Ed., 1944, 71.

DECLINE and RISE of PLASMA VITAMIN A

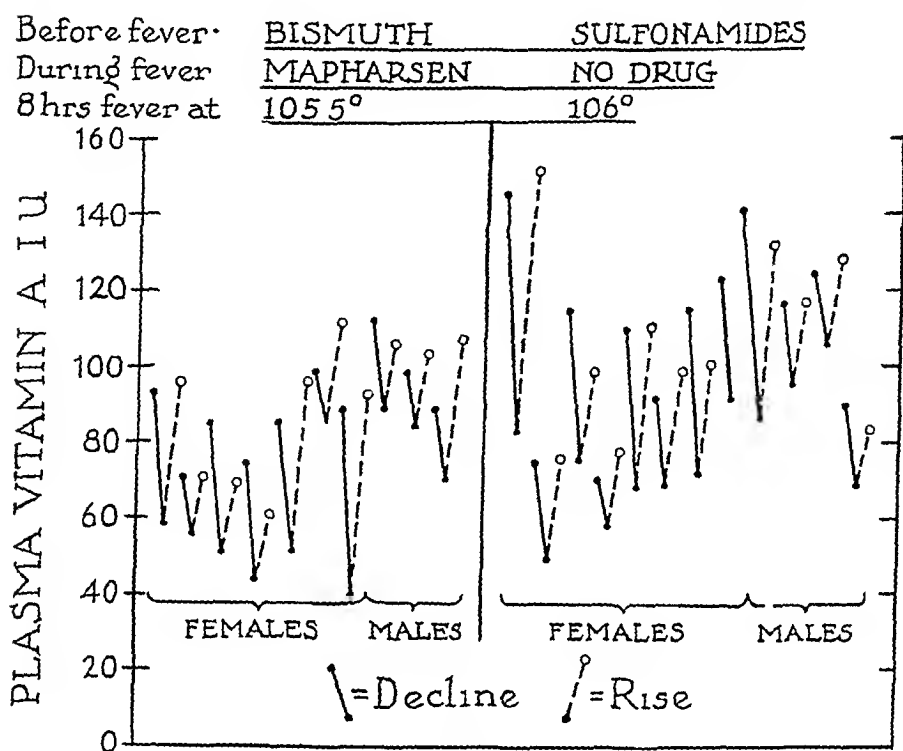


Fig 1

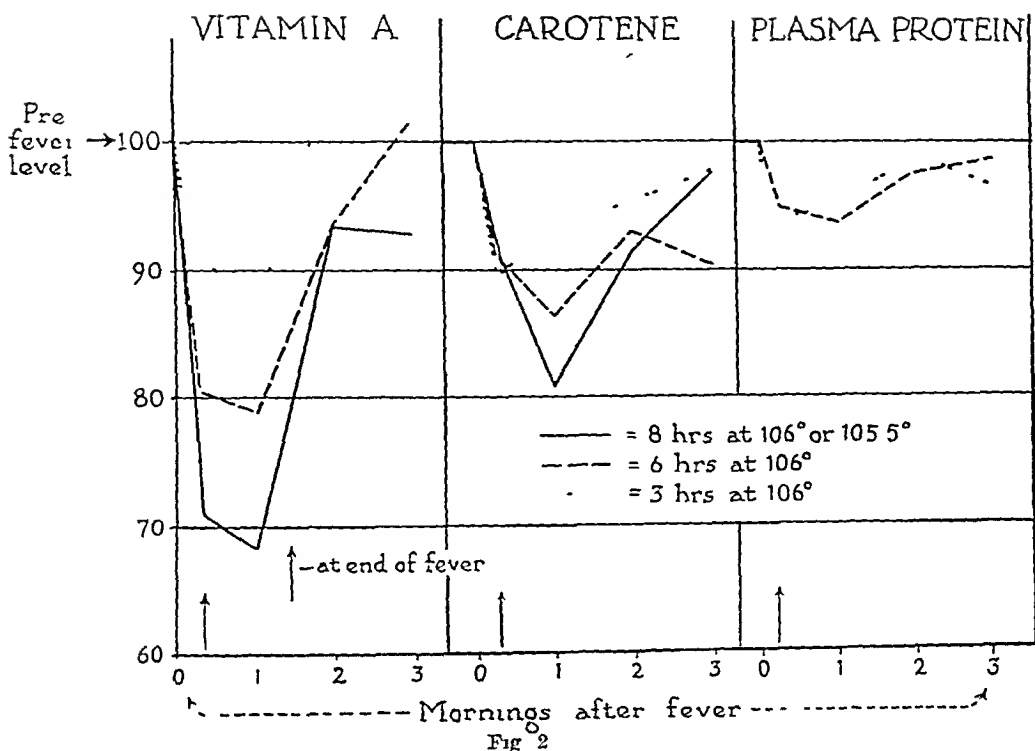
(rectal) by means of physically-induced fever. This depression reveals an identical angle of decline irrespective of the length of fever, as may be seen in Fig 2. The extent of the depression, however, depends on the duration of the fever. The plasma vitamin A drops approximately 30% after 8 hours of fever, 20% after 6 hours, and 10% after 3 hours at 106° F (rectal) (Fig 2). Three hours of fever at 105° F (rectal) combined with 60 mg mapharsen had no influence on the plasma vitamin A. The various chemotherapeutic agents, bismuth subsalicylate, mapharsen, sodium penicillin or sulfathiazole, in the amounts administered before or during fever, had no significant influence on the variation of the plasma vitamin A and carotene.

The maximum depression of the plasma vitamin A was noted after termination of fever-chemotherapy. This level remained ap-

proximately the same until the next morning, 24 hours following the beginning of the fever session. During the second 24 hours, or from 24 to 48 hours from the time the fever session was begun, the plasma vitamin A level rose, and, in 72 hours from the time the fever was inaugurated, the plasma vitamin A had attained levels which approximated the prefever level. The restoration of the plasma vitamin A occurred spontaneously without any special medication or dietary measures.

The plasma carotene followed a pattern similar to that of vitamin A, although there was less depression of the carotene, *viz*, approximately 20% after 8 hours, 15% after 6 hours, and 10% after 3 hours of fever (Fig 2). The restoration of the plasma carotene level was similar to the plasma vitamin A.

Hydremia, as evidenced by the decrease in total plasma protein and hemoglobin levels,



was present but not of sufficient magnitude to account for the decreases in the plasma vitamin A and carotene levels. This is particularly illustrated in the 8- and 6-hour fever-chemotherapy groups where the decrease in the levels was from 3 to 7 times that of the hemoglobin or total plasma protein. Fig 2 shows the changes in plasma protein. The changes in hemoglobin are parallel.

Each of the 22 patients given 8-hour sessions of fever-chemotherapy showed a depression of the plasma vitamin A of more than 15%. A total of 53 patients in the 3 groups were given 6-hour sessions of fever-chemotherapy at the level of 106.0°F (rectal), and all but one patient showed decreases of the plasma vitamin A level at the termination of the fever sessions or on the first morning after fever, as compared to the prefever levels. Five of these patients did have a depression of less than 10%, i.e., within the possible range that might be attributed to the error of the method, for which no clinical explanation can be given. A depression of the plasma vitamin A level was found in all of

the 8 patients subjected to 3-hour fever-chemotherapy at 106.0°F (rectal). One of these patients had a depression of less than 10%. The number of hours the fever was maintained to some degree may account for this factor, as this phenomenon was not observed in the 8-hour fever sessions.

There was no significant difference in the vitamin A levels of those patients who experienced uneventful fever sessions as compared to those who had clinical evidence of dehydration, emesis or low blood pressure during or after fever-chemotherapy.

Four of the 84 patients who received fever-chemotherapy at the level of 105.5° to 106.0°F (rectal) developed mild jaundice with an icterus index† between 14 and 45 units. The plasma vitamin A values of 3 of these pa-

† Icterus index (II) was determined by comparison of the color of the serum against a standard solution of potassium dichromate in a block comparator.¹⁵

¹⁵ Simmons, J. H., and Gentzkow, C. J., *Laboratory Methods of the U. S. Army*, 5th Ed., 1944, 71.

DECLINE and RISE of PLASMA VITAMIN A

Before fever:	BISMUTH	SULFONAMIDES
During fever:	MAPHARSEN	NO DRUG
8 hrs fever at.	105.5°	106°

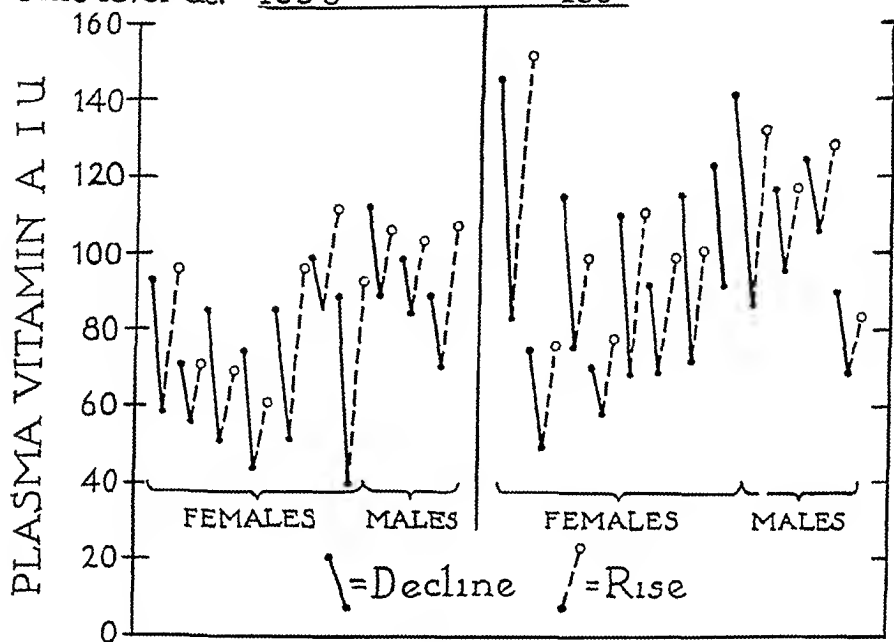


Fig 1

(rectal) by means of physically-induced fever. This depression reveals an identical angle of decline irrespective of the length of fever, as may be seen in Fig 2. The extent of the depression, however, depends on the duration of the fever. The plasma vitamin A drops approximately 30% after 8 hours of fever, 20% after 6 hours, and 10% after 3 hours at 106.0°F (rectal) (Fig 2). Three hours of fever at 105.0°F (rectal) combined with 60 mg mapharsen had no influence on the plasma vitamin A. The various chemotherapeutic agents, bismuth subsalicylate, mapharsen, sodium penicillin or sulfathiazole, in the amounts administered before or during fever, had no significant influence on the variation of the plasma vitamin A and carotene.

The maximum depression of the plasma vitamin A was noted after termination of fever-chemotherapy. This level remained ap-

proximately the same until the next morning, 24 hours following the beginning of the fever session. During the second 24 hours, or from 24 to 48 hours from the time the fever session was begun, the plasma vitamin A level rose, and, in 72 hours from the time the fever was inaugurated, the plasma vitamin A had attained levels which approximated the prefever level. The restoration of the plasma vitamin A occurred spontaneously without any special medication or dietary measures.

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Hydremia, as evidenced by the decrease in total plasma protein and hemoglobin levels,

of events were briefly as follows

Sgt L F was a member of a general hospital medical detachment who had been in consistently good health

May 8 1944 Played baseball with vigor

" 9 Donated 500 cc of blood which was given to patient McG on May 10

" 11 L F reported to sick call. Questionable icterus of sclerae was reported and he was admitted to hospital

" 12 Mild jaundice was present

" 13 Appeared to be average case of infectious hepatitis but during night became actively delirious

" 14 Became comatose and expired

This patient appears to represent an example of naturally acquired infectious hepatitis in which the onset and the appearance of jaundice coincided. The course was unusually brief with fatal outcome.

Pfc McG was admitted to a hospital in Italy April 25, 1944, because of perforating wound of abdominal wall.

May 3, 1944 Transferred to a general hospital in the base area. Progress had been satisfactory.

" 10 Received transfusion of blood from L F

" 18 Serum 5-187 used in this study was obtained

" 19 A series of tests revealed no abnormality

" 21 Complained of lower abdominal pain and discomfort, one loose stool, temp 99°

" 23 Enlargement of cervical lymph nodes

" 24 Serum 5-231 used in this study was obtained

" 25 Occasional needle-like pains in the right upper quadrant. Liver just palpable and very tender

" 27 Liver larger, tender, tip of spleen palpable. Patient felt better

" 29 Patient felt well. Liver smaller and less tender

" 30 Frontal headache, temp 102.6°. Liver edge one f b below costal region, not tender

" 31 Patient felt ill, icteric tinge to sclerae

June 1 Definite icterus. Patient quite ill. Temp 98° to 102.6°. Liver larger and very tender

There had been no history of malaria, previous jaundice or dysentery, no known insect bites. Malarial smear and Kahn test were negative, no ova or parasites were found in stool.

The patient's course was that of a seriously ill person for one week thereafter, but recovery was satisfactory. It seems quite certain that transfusion was the method by which the virus was transmitted, with symptoms 11 days and jaundice 21 days, thereafter.

Materials The histories of the cases and the materials employed in the study were obtained by Colonel M H Barker, MC, AUS, and Captain Frederick G Robbins, MC, AUS, of the 15th Medical General Laboratory in Italy (Colonel V H Cornell, MC, AUS Commanding). The sera were stored in dry ice and shipped in a frozen state by air to Brigadier General S Bayne-Jones, Preventive Medicine Service, Office of The Surgeon General, United States Army, but were in a liquid state at the time of their arrival. They were again frozen in CO₂ ice and transferred, frozen, to this laboratory where they were stored in CO₂ ice until preparation for study.

Subjects The subjects were inmates of the State Prison of Southern Michigan, Jackson, Michigan, who volunteered to participate in the study. Only one case of catarrhal jaundice had been seen in the institution in a period of 9-10 months. From those who offered to take part, the subjects were selected after thorough physical examination. X-ray of the chest was taken in each instance to eliminate those with significant pulmonary or cardiac abnormalities. Examinations of the urine included those for urobilinogen and bilirubin, examination of the blood was made for bilirubinemia. Bromsulfalein retention (5 mg—30 minutes) and Hanger cephalin-cholesterol flocculation tests^{1,2} were carried out for evidence of hepatic dysfunction. Individuals with history of syphilis, positive

¹ Hanger, F M, *J Clin Inv*, 1939, **28**, 261

² Frisch, A W, and Quiligan J J, in press

tients compared to the prefever levels were definitely lower than those of any patient in the same group without jaundice

There was a delay, however, in the spontaneous rise of the plasma vitamin A on the second and third days after fever in those patients who developed jaundice. The reports of others¹⁶ and our own observations point toward the idea that the "recovery rise" of the plasma vitamin A after fever may be an indicator of the return of normal liver function. As long as the liver function is impaired, insufficient vitamin A is released from the liver stores into the circulation. On this basis, therefore, the plasma vitamin A may remain at a low level. Vitamin A is released into the blood stream when the liver regains its normal function and then the plasma vitamin A increases. In those patients in whom the rise of plasma vitamin A was delayed, after fever-chemotherapy clinical jaundice and an elevated icterus index were noted simultaneously with the delay or soon thereafter.

These observations suggest that if the plasma vitamin A is depressed to 50% or less of the prefever level, the development of jaundice may be expected. However, the reverse did not hold true as elevated icterus indices were not necessarily concurrent with extremely low

plasma vitamin A values

Summary In a study of 92 patients it was found that elevation of the body temperature to 105.5° or 106.0°F (rectal) by physically induced fever was followed by a depression of the plasma vitamin A and carotene. The extent of the depression was directly related to the duration of the fever. The course of the plasma vitamin A and carotene was almost identical irrespective of the chemotherapeutic agents (bismuth subsalicylate in oil, mapharsen, sulfathiazole or the sodium salt of penicillin) given either before or during physically-induced fever of equal duration. It is, therefore, justifiable to conclude that elevation of the body temperature is the principal cause of the depression of plasma vitamin A and carotene. At the termination of fever, the plasma vitamin A was nearly at the lowest level. The restoration of the plasma vitamin A level usually occurred by the second day after treatment and took place spontaneously without any special medication or dietary measures. The plasma carotene level showed a pattern similar to that of the plasma vitamin A. The depression of the carotene was generally not as great and occurred more slowly. This behavior of the plasma vitamin A is, to the best of our knowledge, the first biochemical indicator shown to serve as a measurement of the intensity of the action of physically-induced fever on the human organism.

¹⁶ Meyer, K. A., Steigmann, F., Poppei, H., and Walters, W. H., *Arch. Surg.*, 1943, **47**, 26

15299

Demonstration of Infectious Hepatitis Virus in Presymptomatic Period after Transfer by Transfusion *

THOMAS FRANCIS, JR., ARTHUR W. FRISCH, AND J. J. QUILLIGAN, JR.

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The present paper is to report observations upon the presence of the virus of infectious hepatitis in the serum of a patient following the receiving of a transfusion of

blood taken from another individual 48 hours before the onset in the latter of apparently naturally acquired infectious hepatitis.

The circumstances surrounding this series

* This investigation was conducted under the Commission on Influenza, Board for the Investigation and Control of Influenza and other Epidemic

Diseases in the Army, Preventive Medicine Service, Office of the Surgeon General, United States Army.

onset of the preicteric stage of clinical illness. The latter figures were 38, 40 and 43 days, respectively, after inoculation while clinical icterus was noted after intervals of 42, 45 and 47 days. Biochemical tests were correspondingly positive. The marked variation in clinical severity in the 4 individuals receiving the same inoculum was striking. In one, Fd, a rapidly developing disease with fatal termination was encountered, another, Mc, had a prolonged course with a brief relapse, another had quite a mild clinical disease with maintenance of good physical state and good appetite most of the time, the fourth had no detectable disturbance.

One of the second group of subjects, inoculated with serum obtained from the same patient 3 days after the first minimal symptoms and 6 days after the specimen used with group 1, developed symptoms 43 days, and jaundice 46 days, after injection. At the time the other patients were developing hepatitis the 3 additional members of this group had short periods of gastric distress, lassitude, tenderness in right upper quadrant, no jaundice developed and in only one of the 3, Dn, was any disturbance of liver function suggested by laboratory procedures.

Although the subjects were not isolated during the period before the onset of illness no other cases of jaundice occurred in the population.

Discussion. The results of the above observations are of interest from several points of view. It seems quite clear that the disease in the original donor of blood was naturally acquired infectious hepatitis which was transmitted from him by transfusion of blood before the recognized onset of his illness. In turn, the agent was demonstrated in the blood of the recipient 3 days before the onset of any symptoms while biochemical tests were entirely normal, 13 days before the onset of jaundice. The first symptoms noted in the recipient of the transfusion were 11 days after the transfusion and jaundice began 21 days after transfusion.

Both Havens,³ and Neeffe, Stokes and Gel-

lis,⁴ have commented that with serum from cases of naturally acquired infectious hepatitis the interval between administration and onset of jaundice was less than 37 days regardless of whether the material was given by mouth or parenterally. Oliphant,⁵ however, employed for subcutaneous inoculation 0.5 cc of a 1:6 dilution of serum from a case of infectious hepatitis naturally acquired in Italy and induced jaundice in 4 of 11 subjects after intervals of 85-106 days. MacCallum and Bradley⁶ also witnessed incubation periods of 64, 75 and 92 days after subcutaneous inoculation of 1.25 cc of serum from infectious hepatitis. In the present study the average interval before jaundice in the 4 volunteers receiving the equivalent of 0.25 cc of filtered serum subcutaneously was 45 days, a length of time intermediate between the 2 groups of observations.

Havens⁷ states that no appreciable difference in incubation period was observed when doses of 0.01 to 0.5 cc of the same serum from infectious hepatitis was given subcutaneously. Neeffe, Stokes, Reinhold and Lukens⁸ gave 1, 9, 10 and 12 cc, respectively, of icterogenic plasma intravenously to 4 subjects and jaundice occurred at decreasing intervals of 110, 99, 74 and 73 days, thereafter, suggesting some relation to the amount injected. A fifth subject who received 100 cc did not develop jaundice. In the present study the average incubation period was more than twice as long in the volunteers (third passage) as in the transfused patient (second passage) from whom the serum was obtained, although the route was in both instances parenteral. Moreover, there was a relative uniformity in the time of onset of jaundice in the inoculated individuals although the

⁴ Neeffe, J. R., Stokes, J., Jr., and Gelis, S. S., *Am J Med Sci* 1945, **210**, 561.

⁵ Oliphant, J. W., *Pub Health Rep*, 1944, **59**, 1614.

⁶ MacCallum, F. O., and Bradley, W. H., *Lancet*, 1944, **2**, 228.

⁷ Havens, W. P. Jr., *Am J Pub Health*, 1945, **36**, 37.

⁸ Neeffe, J. R., Stokes, J. Jr., Reinhold, J. G., and Lukens, F. D. W., *Am J Clin Inv*, 1944, **23**, S36.

³ Havens, W. P., Jr., *Proc Soc Exp Biol and Med*, 1945, **58**, 203.

TABLE I
Results of Inoculation of Volunteers with Serum from Infectious Hepatitis

Group	Subject	Age	Race	Inoculated	Onset of				
					First possible symptoms days	Definite illness days	Jaundice days	Laboratory evidence	Duration days
1	Fd	24	C	4/13/45	38	38	42	+	8
	Fy	24	W	"	38	40	45	+	21
	Mc	24	W	"	35	43	47	+	34
	Ro	34	W	"	0	0	0	0	0
2	Bn	21	W	4/13/45	42	0	0	0	5
	Dn	22	C	"	*	0	0	0	—
	Gy	25	W	"	45	0	0	0	6
	Lr	30	W	"	43	43	46	+	35

* This patient complained of headache, nausea, vomiting and tenderness in right upper quadrant intermittently from the second day after inoculation for a period of 3 months. Certain laboratory tests were irregularly suggestive between the fifth and ninth weeks after inoculation but neither chemical nor chemical evidence of icterus was obtained.

serological tests or history of previous attacks of jaundice were excluded. Only one of the selected individuals was over 30 years of age.

Inoculations (1) Serum 5-187 was obtained May 18, 1944, from patient McG 8 days after receiving transfusion, 3 days before any symptoms and 13 days before icterus was noted. The serum had been preserved in a tightly stoppered tube by freezing in a CO₂ ice cabinet. On April 10, 1945, the specimen was thawed, 125 cc of serum was mixed with 375 cc of sterile Ringer solution, centrifuged at 1500 r p m for 15 minutes and filtered through a Mandler filter under 9-lb pressure per square inch. Aerobic and anaerobic cultures were bacteriologically sterile after 48 hours. On April 13, 1945, 10 cc of the filtrate of the 1:4 dilution of serum was given subcutaneously to each of 4 volunteers (Fd, Fy, Mc, Ro) with care taken to use individual needles and syringes.

(2) The same procedure was followed with the sample of serum 5-231 obtained from patient McG May 24, 1944, 14 days after transfusion, 3 days after the first of any symptoms, and 7 days before jaundice was clinically detected. On April 13, 1945, 10 cc of the filtrate of the 1:4 dilution of serum was given subcutaneously to each of 4 volunteers (Bn, Dn, Gy, Lr) with individual needles and syringes.

Observation The inoculated individuals were returned to their usual quarters and permitted to continue their usual activities,

including customary work, until any evidence of illness was obtained. During this period they were examined twice weekly by one of the investigators and temperatures and symptoms were recorded. Examination of the urine, including tests for urobilinogen and bilirubin, leukocyte counts, blood bilirubin, bromsulfalein and cephalin-cholesterol flocculation tests were done at 10-day intervals. When any suggestive symptoms or signs were noted the patient was transferred to the institutional hospital where close clinical observations could be maintained. There laboratory examinations were frequently made (with the exception of bromsulfalein) and diets high in carbohydrate and selected protein were instituted. Glucose, sucrose, amino acid concentrates, plasma, vitamin B complex and other fluids were given intravenously in an effort to minimize the course of the disease. All individuals were kept under observation for at least 130 days.

Results In Table I are summarized the results observed in the 2 groups of individuals. Three of the 4 inoculated subcutaneously with serum from McG 8 days after transfusion developed infectious hepatitis and jaundice. The first symptoms which seemed even in retrospect to be of significant relationship were noted 35, 38 and 38 days, respectively, after inoculation. In only the case of Mc was there an appreciable interval, 8 days, between the first symptoms noted and what appeared to be the actual

of energy-yielding substrates. The effectiveness of a substance in restoring amplitude was considered a measure of its ability to serve as a source of energy for smooth muscle contraction. More recently, Feldberg and Solandt,⁴ and Feldberg,⁵ in extending the work of the earlier investigators, have arrived at the same conclusions.

Except for the studies cited, the procedure introduced by Rona and Neukirch has not been utilized for the investigation of the intermediary metabolism of smooth muscle. If their assumption is correct, that the restoration of amplitude is evidence of the ability of a substance to provide energy for smooth muscle contraction, then the method would appear to be unusually well suited for such studies. The restoration of contractility would comprise a more exacting and reliable criterion for the evaluation of a substrate, than is provided by a rise in oxygen consumption alone. The latter criterion is frequently un dependable, the former furnishes direct evidence that the energy provided by a substance under study is actually utilizable for smooth muscle contraction.

In the present study the method has been employed for a more extensive investigation of potential energy-yielding substrates, selected on the basis of modern concepts of intermediary metabolism, and particular attention has been given to the possible usefulness of fatty acids.

Methods The standard preparation consisted of a fresh segment, approximately 4 cm long, from the upper portion of rabbit small intestine. The segment, with its lumen open, was suspended in a 50 cc muscle chamber, and attached to an isotonic muscle lever. The lever exerted a tension of 4 g and recorded the contractions of the longitudinal muscle on a kymograph. The intestinal segment was bathed in glucose-free Krebs-Henseleit solution,⁶ maintained at 37.5°, and aerated with 95% O₂ - 5% CO₂ to obtain a

pH of 7.4

Prior to testing each substance, the spontaneous rhythmic contractions of the longitudinal smooth muscle were allowed to decrease to about 1/10 to 1/20 of the original amplitude. This usually required less than 1 hour and introduced no change in contraction frequency. At this point the test substance, dissolved in a small quantity of Krebs solution, was added to the muscle chamber. All acids were used in the form of sodium salts. For each substance tested, the minimal concentration was determined which brought about the maximal amplitude of contraction of which that substance was capable. This increase in amplitude was then compared on a percentage basis with the maximal change which resulted from the addition of glucose to the same intestinal preparation.

Results The record of a typical experiment is reproduced in Fig. 1. It illustrates the gradual decrease in amplitude and average tonus which takes place in the absence of substrate as well as the restoration of both on the addition of glucose (200 mg %). When the amplitude had fallen to about 1/10 to 1/20 of the original value. The amplitude and average tonus began to increase within a minute of the addition of glucose and the maximal amplitude was reached within 10 minutes, and maintained thereafter. In this experiment, the maximal amplitude following glucose was greater than that initially exhibited, in other experiments, the amplitude attained after the addition of glucose was either equal to, or greater than, but never less than, the original amplitude. Not discernible in the tracing because of the reduction in size, are the number of contractions per minute given by the intestinal segment and the constancy of this frequency throughout the experiment, despite marked changes in amplitude. Under our experimental conditions, the contractions averaged 17 per minute with most of the intestinal segments used. This uniformity of contraction rate throughout the experiment simplified the comparison of the relative effectiveness of different substrates in providing energy for contraction, since the only variable introduced consisted of a change in amplitude.

⁴ Feldberg, W., and Solandt, O. M., *J. Physiol.*, 1942, **101**, 137.

⁵ Feldberg, W., *J. Physiol.*, 1943, **102**, 108.

⁶ Krebs, H. A. and Henseleit, K., *Z. f. Physiol. Chem.*, 1932, **210**, 33.

clinical responses varied considerably in severity. The difference strongly suggests that the amount of effective virus in the inoculum had a significant influence upon the result. If this should not be the case one might suggest that a variation in the virus had occurred during its 2 passages by parenteral inoculation in human subjects. In any case the data illustrate the difficulty in classifying infectious hepatitis on the basis of the incubation period after parenteral injection of icterogenic serum. The relative uniformity in the time of onset of jaundice in the inoculated individuals indicates, on the other hand, that the variation in severity of the clinical courses following the same inoculum is related to individual susceptibility.

The events demonstrate how, with virus in the blood well before the onset of symptoms, infectious hepatitis may be transmitted by blood products derived from individuals in whom the presence of the virus would be entirely unsuspected.

Summary A transfusion of blood from an individual in the incipient stage of in-

fectious hepatitis elicited the first symptoms in the recipient after 11 days and jaundice after 21 days.

By subcutaneous inoculation of human volunteers the virus was demonstrated in the serum of the recipient on the eighth day after transfusion which was 3 days before the first symptoms and 13 days before the onset of jaundice. Virus was also demonstrated in another specimen of serum taken 6 days later, or 3 days after first symptoms.

The incubation period as measured by the onset of jaundice in the 4 subjects who had definite illness was 42 to 47 days, while the clinical response varied greatly in severity.

The significance of the results is discussed.

This investigation was made possible by the cooperation of Dr. Garrett Heyns, Director of Corrections, State of Michigan, and Dr. W. B. Huntley, Medical Director of the State Prison of Southern Michigan, Jackson, Michigan, and his staff, to whom the authors wish to express their appreciation for constant assistance. They wish also to express their indebtedness to the volunteers who participated so willingly and cooperatively in the study.

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Sources of Energy for Intestinal Smooth Muscle Contraction*†

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The rhythmic contractions of a segment of rabbit small intestine, suspended in glucose-free Tyrode solution, were found by Rona and Neukirch^{1,2,3} to undergo a gradual and

progressive decrease in amplitude, at which time, the addition of certain substances (glucose, mannose, pyruvic, lactic, acetic, butyric, β -hydroxybutyric and oxaloacetic acids) served to increase and maintain the amplitude of contraction, the extent of the increase varying with the substance added. They attributed the progressive decrease in the height of the contractions in the absence of glucose to gradual depletion of the endogenous stores

* The work described in this paper was done under a contract recommended by the Committee on Medical Research, between the Office of Scientific Research and Development and Cornell University Medical College.

† We are indebted to Dr. David Rittenberg for samples of enanthic and pelargonic acids, and to Dr. Severo Ochoa for the α -ketoglutaric acid used in this study.

¹ Neukirch, P., and Rona, P., *Arch. f. Physiol. (Pflüger's)*, 1912, **144**, 555.

² Rona, P., and Neukirch, P., *Arch. f. Physiol. (Pflüger's)*, 1912, **146**, 371.

³ Rona, P., and Neukirch, P., *Arch. f. Physiol. (Pflüger's)*, 1912, **148**, 273.

of energy-yielding substrates. The effectiveness of a substance in restoring amplitude was considered a measure of its ability to serve as a source of energy for smooth muscle contraction. More recently, Feldberg and Solandt,⁴ and Feldberg,⁵ in extending the work of the earlier investigators, have arrived at the same conclusions.

Except for the studies cited, the procedure introduced by Rona and Neukirch has not been utilized for the investigation of the intermediary metabolism of smooth muscle. If their assumption is correct, that the restoration of amplitude is evidence of the ability of a substance to provide energy for smooth muscle contraction, then the method would appear to be unusually well suited for such studies. The restoration of contractility would comprise a more exacting and reliable criterion for the evaluation of a substrate, than is provided by a rise in oxygen consumption alone. The latter criterion is frequently undependable, the former furnishes direct evidence that the energy provided by a substance under study is actually utilizable for smooth muscle contraction.

In the present study the method has been employed for a more extensive investigation of potential energy-yielding substrates, selected on the basis of modern concepts of intermediary metabolism, and particular attention has been given to the possible usefulness of fatty acids.

Methods The standard preparation consisted of a fresh segment, approximately 4 cm long, from the upper portion of rabbit small intestine. The segment, with its lumen open, was suspended in a 50 cc muscle chamber, and attached to an isotonic muscle lever. The lever exerted a tension of 4 g, and recorded the contractions of the longitudinal muscle on a kymograph. The intestinal segment was bathed in glucose-free Krebs-Henseleit solution,⁶ maintained at 37.5°, and aerated with 95% O₂ - 5% CO₂ to obtain a

pH of 7.4

Prior to testing each substance, the spontaneous rhythmic contractions of the longitudinal smooth muscle were allowed to decrease to about 1/10 to 1/20 of the original amplitude. This usually required less than 1 hour and introduced no change in contraction frequency. At this point, the test substance, dissolved in a small quantity of Krebs solution, was added to the muscle chamber. All acids were used in the form of sodium salts. For each substance tested, the minimal concentration was determined which brought about the maximal amplitude of contraction of which that substance was capable. This increase in amplitude was then compared on a percentage basis with the maximal change which resulted from the addition of glucose to the same intestinal preparation.

Results The record of a typical experiment is reproduced in Fig. 1. It illustrates the gradual decrease in amplitude and average tonus which takes place in the absence of substrate as well as the restoration of both on the addition of glucose (200 mg %) when the amplitude had fallen to about 1/10 to 1/20 of the original value. The amplitude and average tonus began to increase within a minute of the addition of glucose, and the maximal amplitude was reached within 10 minutes, and maintained thereafter. In this experiment, the maximal amplitude following glucose was greater than that initially exhibited; in other experiments, the amplitude attained after the addition of glucose was either equal to, or greater than, but never less than, the original amplitude. Not discernible in the tracing because of the reduction in size, are the number of contractions per minute given by the intestinal segment and the constancy of this frequency throughout the experiment, despite marked changes in amplitude. Under our experimental conditions, the contractions averaged 17 per minute with most of the intestinal segments used. This uniformity of contraction rate throughout the experiment simplified the comparison of the relative effectiveness of different substrates in providing energy for contraction, since the only variable introduced consisted of a change in amplitude.

⁴ Feldberg, W., and Solandt, O. M., *J. Physiol.*, 1942, **101**, 137.

⁵ Feldberg, W., *J. Physiol.*, 1943, **102**, 108.

⁶ Krebs, H. A., and Henseleit, K., *Z. f. Physiol. Chem.*, 1932, **210**, 33.

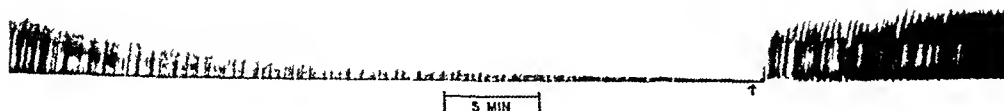


Fig 1

Kymograph tracing showing decrease in amplitude of intestinal contractions in glucose free solution, and restoration of contraction amplitude on addition of glucose (at arrow) to provide a concentration of 200 mg %

The second step in the experimental procedure—that is, the comparison of the relative effectiveness of different substrates in supporting smooth muscle contractions—is illustrated in Fig 2. The results obtained with 4 substrates are expressed in semi-diagrammatic fashion, each contraction wave representing approximately 17 contraction waves in the actual kymograph tracing. With the amplitude after addition of glucose as the standard of reference, the relative effectiveness of the other substrates is as follows: pyruvic acid, 100%, butyric acid,

50%, and enanthic acid, 20%.

In Tables I and II, are assembled the results obtained with a number of substances which proved capable of restoring, in varying degrees, the amplitude of smooth muscle contraction. In Table I, the height of the response is compared with that produced by glucose, and, for each substance, represents the maximal response which it can elicit. Table II lists the minimal concentration of each substrate required to bring about its individual maximal increase in amplitude.

Table III lists those substances which failed

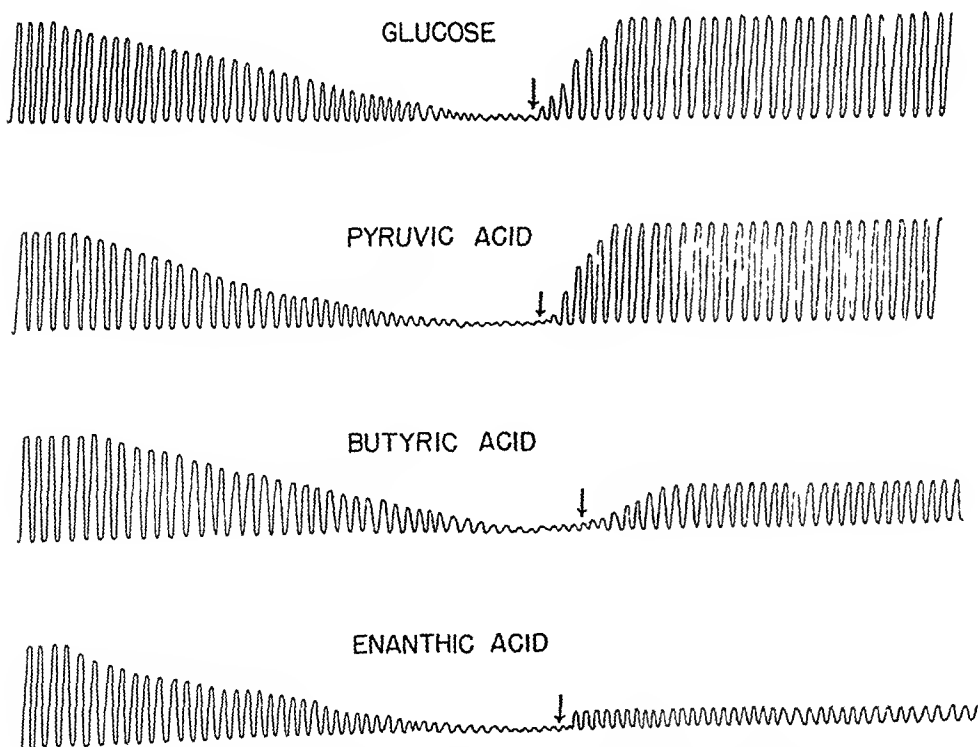


Fig 2

Semi diagrammatic representation, based on kymograph tracings, illustrating relative effectiveness of glucose, pyruvic, butyric, and enanthic acids in restoring amplitude of contraction of intestinal smooth muscle after prior exposure to a glucose free solution

TABLE I
Substances which Increase Amplitude of Contraction

Substance	Response compared with that of glucose
	%
Glucose	100
Mannose	70-90
Acetic acid	100
Pyruvic acid	100-110
Oxaloacetic acid	75-100
d,l Lactic acid	20-40
Acetoacetic acid	20-30
Butyric acid	40-60
Caproic acid	40-60
Caprylic acid	40-60
Pelargonic acid	35-45
Enanthic acid	15-20
Valeric acid	5-10

to increase the amplitude of contraction. Each was tested over a range of concentration extending from 10 to 100 mg % and found to be without influence on the gradual reduction in tone and amplitude of contraction characteristic of the preparation in the absence of substrate. There were 2 exceptions: acetaldehyde and glyceraldehyde, in concentrations of 100 mg %, exerted a marked inhibitory effect, as evidenced by the rapid and virtually complete cessation of all contractile activity. The inhibition induced by acetaldehyde could not be overcome by the subsequent addition of glucose, pyruvate, acetate or lactate, whereas that caused by glyceraldehyde could be overcome by pyruvate, acetate, or any of the fatty acids listed in Table I, but not by glucose or lactate. In the case of citric acid, which removes Ca ions from solution to form a weakly dissociable

TABLE II
Minimal Concentrations Necessary for Maximal Increases in Amplitude of Contraction

Substance	Minimal concentration in mg %
Glucose	100-125
Mannose	125-150
d,l Lactic acid	20-30
Oxaloacetic acid	20-30
Acetic acid	10-15
Pyruvic acid	10-15
Butyric acid	4-5
Valeric acid	3-4
Caproic acid	2-3
Enanthic acid	1-2
Caprylic acid	0.5-0.75
Pelargonic acid	0.5

TABLE III
Substances which Fail to Increase Amplitude of Contraction

Galactose	Propionic acid
Fructose	Hydroxyacetic acid
Glycerol	Citric acid
Glucose 1 phosphate	Succinic acid
β Phosphoglyceric acid	Fumaric acid
Glycerol	α Ketoglutaric acid
d,l Glyceraldehyde	Glycine
Acetaldehyde	l(+)-Alanine
Ethanol	l(-)-Aspartic acid
Acetone	l(+)-Glutamic acid

(Ca citrate)⁻ complex, the precaution was taken to add sufficient Ca ions (as CaCl₂) to replace those removed, in order to avoid changes in muscle tonus which would otherwise have resulted from a deficiency of Ca ions in the medium.

Fig. 3 is a graphic presentation of the maximal increases in amplitude observed with the series of fatty acids from acetic through pelargonic. The greatest increase, which equalled that given by glucose, was obtained with acetic acid. The remaining even-carbon fatty acids (butyric, caproic and caprylic) all produced an identical increase in amplitude, which was approximately 50% of that of acetic acid. On the other hand, the effects of the odd-carbon series of fatty acids were strikingly different. Propionic acid was devoid of effect. The changes in amplitude induced by the other members of the odd-carbon series were less (valeric, enanthic and pelargonic) than those obtained with even-carbon fatty acids, and differed in extent with each compound. The magnitude of response increased as the chain-length increased. Thus the approximate increase with valeric acid was 10% with enanthic acid 20%, and with pelargonic acid 40% of that observed with acetic acid.

Experiments of a preliminary character have been carried out to investigate the mechanisms responsible for the differences exhibited by odd- and even-carbon fatty acids. If β -oxidation takes place in smooth muscle, as our experiments suggest, the smaller fragments resulting from this type of degradation of the even-carbon fatty acids would all be utilizable for muscle contraction, whereas the odd-carbon fatty acids would leave a non-utilizable propionic acid residue. Since the

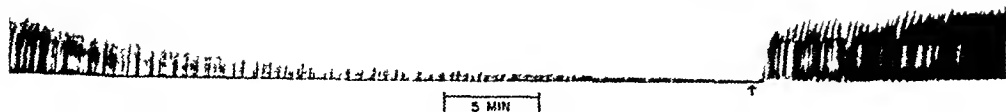


Fig 1

Kymograph tracing showing decrease in amplitude of intestinal contractions in glucose free solution, and restoration of contraction amplitude on addition of glucose (at arrow) to provide a concentration of 200 mg %

The second step in the experimental procedure—that is, the comparison of the relative effectiveness of different substrates in supporting smooth muscle contractions—is illustrated in Fig 2. The results obtained with 4 substrates are expressed in semi-diagrammatic fashion, each contraction wave representing approximately 17 contraction waves in the actual kymograph tracing. With the amplitude after addition of glucose as the standard of reference, the relative effectiveness of the other substrates is as follows: pyruvic acid, 100%, butyric acid,

50%, and enanthic acid, 20%.

In Tables I and II, are assembled the results obtained with a number of substances which proved capable of restoring, in varying degrees, the amplitude of smooth muscle contraction. In Table I, the height of the response is compared with that produced by glucose, and, for each substance, represents the maximal response which it can elicit. Table II lists the minimal concentration of each substrate required to bring about its individual maximal increase in amplitude.

Table III lists those substances which failed

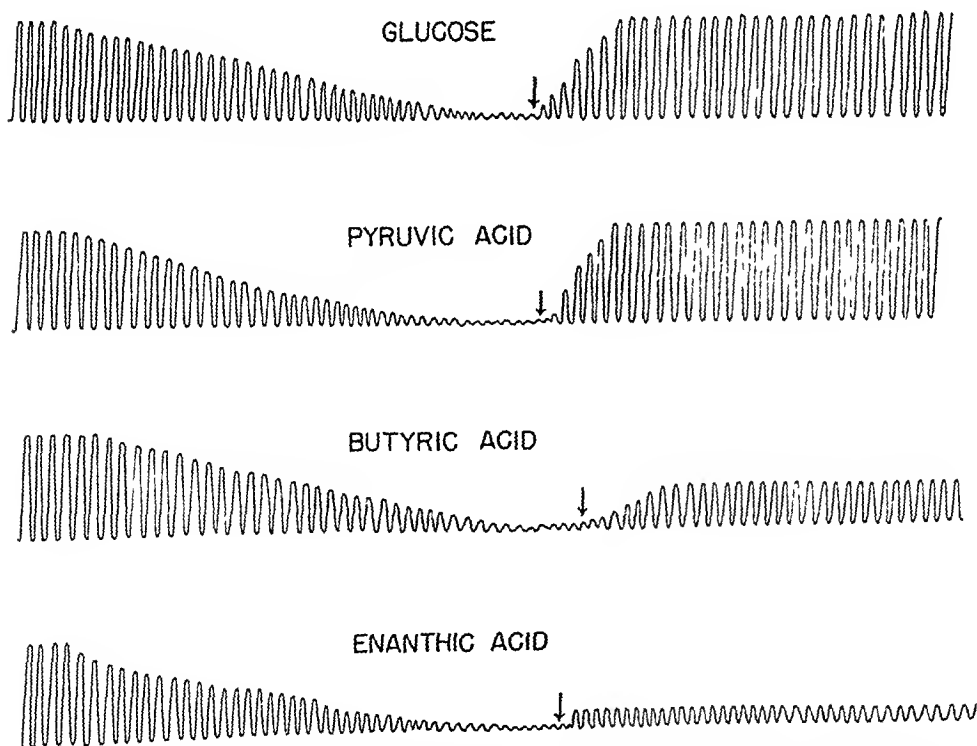


Fig 2

Semi diagrammatic representation, based on kymograph tracings, illustrating relative effectiveness of glucose, pyruvic, butyric, and enanthic acids in restoring amplitude of contraction of intestinal smooth muscle after prior exposure to a glucose free solution.

tors to follow the addition of fructose to a glucose-free medium. We also found mannose to be 70-90% as effective as glucose, in contrast with the 25-30% effectiveness which they observed. This latter discrepancy may be due to the fact that they did not use maximal increases in amplitude as the basis for comparison.

The excellent substrate effect of pyruvic acid is in accord with the Meyerhof cycle. The smaller effect produced by lactate is of interest. The lack of effectiveness of glycogen, glucose-1-phosphate, and β -phosphoglyceric acid need not be considered evidence against the occurrence of the Meyerhof cycle in smooth muscle since it is questionable whether the muscle cells are permeable to these substances. The same reservation holds for the ineffectiveness of glucose-6-phosphate and fructose-1,6-diphosphate, reported by Feldberg.⁵

3 Acetic Acid This proved to be an excellent substrate, producing contraction amplitudes equal to glucose and pyruvate. This is compatible with recent observations on the utilization of acetate by a variety of tissues. The pathway for the oxidation of acetic acid in smooth muscle remains obscure; it does not appear to be oxidized to hydroxyacetic acid, since the latter had no substrate effect.

4 The Krebs Tricarboxylic Acid Cycle Of the acids postulated in this cycle the following were tested and found ineffective: citric, α -ketoglutaric, succinic and fumaric. Oxaloacetic acid exerted a good substrate effect. However, the exceptional behaviour of oxaloacetic acid prompts the suggestion that it may have exerted its effect only after decarboxylation to the readily oxidizable pyruvic acid. The ineffectiveness of succinic acid is in accord with *in vitro* metabolic experiments with strips of dog intestinal smooth muscle (unpublished data). In these experiments carried out at reduced O_2 tensions, the increased O_2 uptake in the presence of succinic acid failed to increase the concentrations of phosphocreatine and adenylypyrophosphate, the high energy phosphates essential for muscle contraction. These results provide no support for the participation of the postulated tricarboxylic acid cycle in the

activity metabolism of smooth muscle and, by the same token, would exclude the cycle as a pathway for the oxidation of pyruvic and acetic acids.

5 Amino Acids In conformity with the earlier observations of Neukirch and Rona,² the amino acids studied were found to exert no substrate activity. This was of particular interest with respect to alanine and aspartic acid, since their corresponding keto-acids, pyruvic and oxaloacetic, are extremely effective. Apparently, intestinal smooth muscle is unable to deaminate these amino acids to their keto forms.

6 Ketone Bodies Neukirch and Rona² had previously reported a small increase in amplitude with β -hydroxybutyric acid. We have observed an effect of similar magnitude with acetoacetic acid, but none with acetone. Should the lack of utilization of acetone prove to be a general phenomenon, it might provide an explanation for the readiness with which acetone appears in the urine even with mild disturbances in fat metabolism.

7 Fatty Acids The difference in the effects observed with odd- and even-carbon fatty acids strongly suggests that β -oxidation takes place in intestinal smooth muscle. Thus, butyric, caproic and caprylic, which can all be completely degraded to 2-carbon fragments, are all equally effective as substrates. On the other hand, propionic acid, which is not susceptible of β -oxidation, is ineffective, and the remaining odd-carbon fatty acids studied, all of which would leave a propionic acid residue on β -oxidation, have a smaller effect than those of the even-carbon series.

An explanation for the identical effects of the even-carbon fatty acids may be that they all undergo β -oxidation at the same enzyme surface, and that, once this surface is saturated with any one of them, β -oxidation then proceeds at the same rate. Support for this hypothesis is provided by the fact that once a maximal increase in amplitude is induced by any one of the series, the subsequent addition of another results in no further increase.

Our experimental results also permit a tentative explanation for the smaller effects of the odd-carbon acids (valeric, enanthic, pelargonic) as well as for the increase in

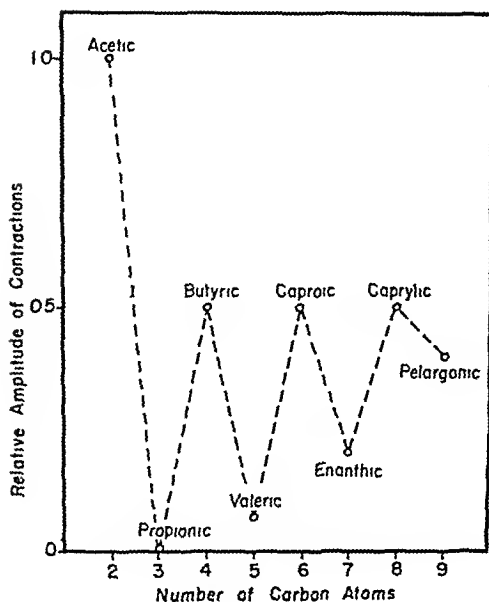


Fig 3

Relative effectiveness of odd and even carbon fatty acids in restoring amplitude of contraction of intestinal smooth muscle following prior exposure to glucose free solution

propionic residue would be formed at the surface of the postulated β -oxidation enzyme, it might conceivably compete for this surface with the unaltered fatty acid or its oxidizable fragments. This concept was explored by adding propionic acid in concentrations of 10-30 mg % to muscle preparations contracting under the influence of butyric or caprylic acid, in concentrations of 50 and 0.75 mg % respectively. In each instance, 30 mg % of propionic acid served to reduce the amplitude of contraction by about 80%. This inhibition, however, could subsequently be overcome by doubling the concentrations of butyric or caprylic acid.

The observation was also made that relatively high concentrations of enanthic, caprylic and pelargonic acids completely inhibited smooth muscle contraction. The effective concentrations varied with different intestinal preparations and were as follows: enanthic 50-200 mg %, caprylic 25-150 mg %, and pelargonic 20 mg % (1 experiment). This inhibition was not overcome by the addition of glucose, pyruvic acid, or acetic acid. However, it proved to be a reversible inhibi-

tion, since contractility was restored when the fatty acid was washed out and replaced with fresh Krebs solution.

Discussion 1 Mode of Action of Substances Which Restore Contractility Those substances which restore contraction amplitude may act either as substrates or pharmacological agents. All the evidence so far accumulated, although indirect, appears to favor the substrate concept. Rona and Neukirch¹ observed a reduction in glucose and mannose content during a 2½-hour period of active contractions. More specific evidence against a pharmacological action was provided by Feldberg and Solandt.⁴ The addition of acetylcholine during a period of diminished activity in a glucose-free Tyrode solution produced a relatively small increase in tonus, sustained for less than a minute. However, following the restoration of amplitude by glucose or pyruvate, acetylcholine produced a marked and well-sustained increase in tonus. From this it was inferred that glucose and pyruvate do not increase smooth muscle activity by increasing the production of acetylcholine, and that this agent could exert its pharmacological effect only when energy for contraction was provided by appropriate substrates. These experiments have been confirmed and extended in our laboratory, with the observation that large and sustained increases in tonus may also be obtained with acetylcholine after the restoration of amplitude by fatty acids, such as acetate and butyrate. Although this evidence is indirect, we favor the substrate concept over the pharmacological, particularly in view of the conformity of much of the data obtained with prevailing views of intermediary metabolism. The discussion of our results has therefore been based on the concept that the capacity of a substance to augment and sustain contraction amplitude is derived from its ability to provide energy for smooth muscle contraction.

2 Carbohydrate Series Of the hexoses studied only glucose and mannose yielded energy for contraction, galactose and fructose were ineffective. In 2 respects our findings differ from those of Feldberg and Solandt.⁴ We failed to find the increase in reactivity to acetylcholine reported by these investiga-

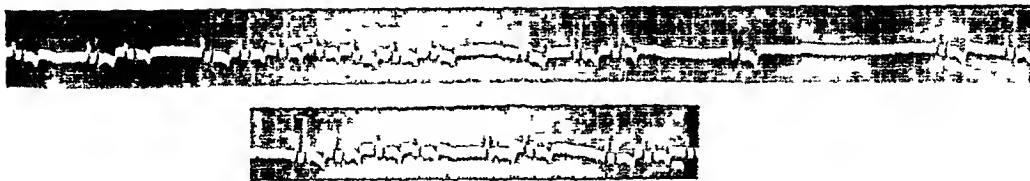


Fig 1a and b Sinuauricular block following subepicardial injection of strophosid at the lower end of the sinus node

in patients with arteriosclerosis of the sinus node arteries is also to be expected. It is difficult to explain, however, the sinoauricular block which follows the administration of digitalis or the form which is occasionally seen in patients with myocardial involvement. The sinus node represents a relatively large structure with a length of more than 25 mm, it is united with the common auricular fibers by innumerable junctional fibers which spread in all directions. Furthermore one must assume that all specific fibers of the sinus node are able to form automatic stimuli. It is hard to conceive how any but a very extensive process could lead to a sinoauricular block; the simultaneous periodical block of the sinoauricular conduction in all junctional fibers due to digitalis action seems impossible.

Therefore for many years Lewis spoke only of "sinoauricular block, so-called"¹ and Clerc discusses le blocage sinoauriculaire ou suppose tel.²

In complete accord with these facts was the experience that in order to obtain sinoauricular block experimentally it is necessary to isolate the sinus node on all 4 sides³ to clamp the sinus node artery and thus damage the tissue of the whole node⁴ or to inject pituitary extracts which cause coronary spasm.⁵ All experimentators who have tried to produce a sinoauricular block agree that this disturbance cannot be easily created.

In experiments which were performed for other purposes we registered electrocardio-

grams showing sinoauricular block. The method by which these tracings were obtained seems worth reporting because it may serve to explain some of the clinical cases of sinoauricular block.

Experimental Results In experiments in which extrasystoles were induced by the local application of digitalis or strophanthin to the sinus node area,⁶ the subepicardial injection of these drugs led in some instances to immediate cardiac standstill and to temporary arrhythmias. These disturbances appeared so soon after the application that it seemed unlikely that they are due to a specific effect of the injected drugs. It was actually found that hypertonic solutions of barium, sodium or calcium chloride evoked the same effect when injected.

Dogs were used in all experiments. During anesthesia with morphine and nembutal the heart was exposed while artificial respiration was started. The subepicardial injection was done with a fine needle, attached to a tuberculin syringe. The amount injected never exceeded 0.1 cc. The epicardium always became detached at the site of injection and was visible as a grey covering over a small vesicle. The arrhythmias could be initiated from all areas of the sinus node but they appeared most often if the injection was made at the lower end of the node. In some experiments the same disturbances of rhythm were also obtained when the injection was made a few millimeters beyond the sinus node area toward the appendix of the right auricle.

Fig 1a was obtained in an experiment in which strophosid was injected at the lower end of the sinus node. A regular sinus rhythm existed with a rate of 127-136, that is the single cycles measured between 0.44

¹ Lewis T, *The mechanism and graphic registrations of the heart beat* London, 1925

² Clerc A *Les arythmies en clinique* Paris 1925

³ Cohn A E, Kessel L and Mason H H, *Heart* 1911 12, 3, 311

⁴ Scherf D, *Z f d ges exp Med* 1927, 57 188

⁵ Resnik W H, *Arch Int Med*, 1925, 36, 788

⁶ Scherf, D *Exp Med and Surg*, 1944, 2, 70

amplitude with the increase in chain-length. The β -oxidation of odd-carbon fatty acids should in every instance leave a non-utilizable propionic acid residue. Propionic acid has been found to reduce the effectiveness of even-carbon fatty acids as substrates, an inhibition which can be overcome by raising the concentration of the utilizable fatty acid. This suggests the competition of propionic acid for the surface of the β -oxidation enzyme. With the odd-carbon series, the competition between the propionic residue and the unaltered acid and its β -oxidizable fragments, would explain the smaller effects of this series. This competition should become less effective as the chain-length increased, since the β -oxidizable portion would progressively increase relative to the propionic acid residue.

One other factor may also contribute in part to the greater effectiveness of the odd-carbon fatty acids of longer chain-length. It was observed, for the whole series of fatty acids, that, as the chain-length increased, progressively smaller concentrations were required to produce maximal amplitude effects. An explanation for this finding may reside in the greater affinity of the longer-chain fatty acids for the β -oxidation enzyme surface, a concept which derives support from the increase in the adsorption coefficients of fatty acids at surfaces, as chain-lengths increase. With respect to the odd-carbon

series, such an affinity relationship would act to the advantage of the longer-chain members in their competition with propionic residues.

No information is at present available as to the usefulness of higher fatty acids such as oleic, palmitic, and stearic, as substrates for smooth muscle contractility, because of the extremely low solubility of their sodium salts in Krebs solution. They were ineffective in dilute suspensions.[†]

Summary A number of substances have been studied with respect to their influence on the contraction of excised segments of rabbit small intestine. The results have been discussed in relation to present concepts of intermediary metabolism. Particular attention has been given to fatty acids, from propionic through pelargonic. With the exception of propionic, all appear to provide energy for smooth muscle contraction, and the characteristics of their behaviour were compatible with the concept of β -oxidation.

[†] Since this manuscript was submitted oleic acid has been found to partially restore contraction amplitude. It was introduced as a sodium oleate sol (100 mg %) in water along with enough NaCl to maintain isotonicity. The effective concentration of oleic acid could not be determined because of its partial precipitation.

We wish to acknowledge the technical assistance of Mathilda Fischl, Alice Kratoch, and Rita Briff.

15301

Experimental Sinoauricular Block *

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From the Medical Department, New York Medical College

Sinoauricular block is a rare disturbance either of stimulus formation in the sinus node or of transmission of the stimulus to the auricle. In analogy with tracings obtained in auriculoventricular conduction disturbances, sinoauricular block is diagnosed whenever,

with a regular sinus mechanism, pauses occur without evidence of auricular activity. These pauses are more or less approximate multiples of a normal period.

A disturbance of stimulus formation in the sinus node or of stimulus conduction in the sinoauricular junctional fibers is understandable during vagus stimulation or carotid pressure, the occurrence of sinoauricular block

* The expenses of this investigation were defrayed by a grant from the United Hospitals Fund.

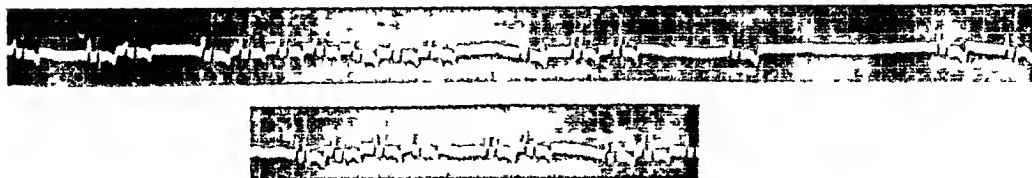


Fig 1a and b Sinoauricular block following subepicardial injection of strophosid at the lower end of the sinus node

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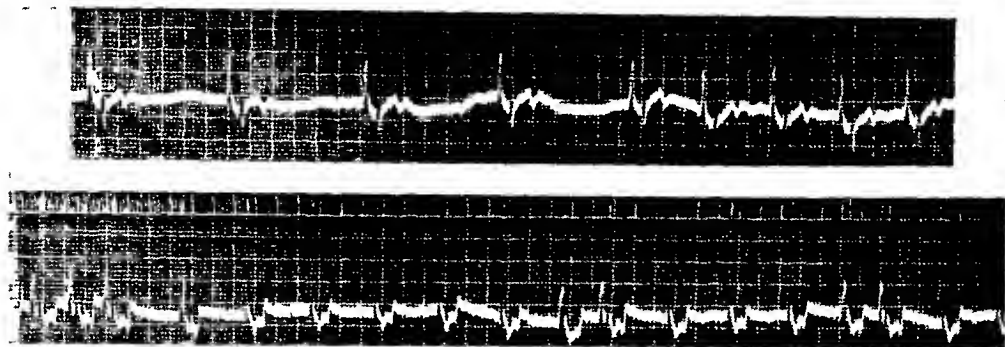


Fig 2 (top) 2:1 sinoauricular block after injection of a 2% solution of sodium chloride over lower end of sinus node

Fig 3 (bottom) Sinus block and an irregular rhythm after injection of 10% solution of sodium chloride over the middle portion of the sinus node

and 0.47 second. Immediately following the injection, pauses occurred in the otherwise regular rhythm. Fig 1a was registered about 10 seconds later. After the first and the third beats in Fig 1a pauses appear, which measure 0.90 second, that is, they are equal to 2×0.45 second. The third pause in the tracing is 1.06 seconds long but it is terminated by an auricular beat with a different P-wave, originating presumably in a different focus. The fourth pause in the tracing is 1.40 seconds long (3×0.47 second), and the last one measures 2.42 seconds (5×0.48 second). About 2 minutes later, with sinus periods of 0.44 to 0.48 second (Fig 1b) the pauses were 0.88 and 0.96 second long, exactly twice the length of a normal period. From the length of these pauses and from their comparison with the other sinus periods, the conclusion is justified that the tracings exhibit sinoauricular block.

In some of the other experiments a marked bradycardia appeared following the injection and the rate then suddenly doubled. Thus the tracing of Fig 2 was recorded after the injection of 0.1 cc of a 2% solution of sodium chloride into the lower region of the sinus node, the P-waves in this experiment were unusually tall and they are therefore followed by pronounced Ta-waves. The length of the first 4 long periods measures 1.26, 1.25, 1.24, and 1.23 seconds. The following short periods measure 0.67, 0.66, 0.66, and 0.64 second. It is well known from clinical and from experimental sinoauricular block that the long period caused by the block is

usually shorter than 2 normal periods.

The effect of the subepicardial injection of a 10% solution of sodium chloride over the middle of the sinus node is visible in Fig 3. The tracing was obtained a few seconds after the injection. The length of the first 2 periods at the beginning of the tracing measures 0.36 and 0.34 second. The period following has a length of 0.72 second (2×0.36 second). Then for 3.60 seconds (10×0.36 second) no P-waves are visible and atrioventricular rhythm prevails. Following this long cessation of the normal sinus mechanism a sinus period of 0.38 second can be measured. The next long pause measures 2.34 seconds (6×0.39 second) and the last short one is 0.38 second. After the disappearance of the sinus block, injection with Digilant C was repeated over the same area and the same result was obtained.

In the majority of cases the disturbance of the activity of the sinus node appeared so quickly that the end of the first (and usually longest) standstill could just be recorded after the injection was completed. In other experiments the arrhythmia appeared after about one minute, and only exceptionally a little later. The disturbance never lasted longer than 6 minutes.

In view of the appearance in an otherwise regular rhythm of pauses having a length which is a multiple of a normal period and during which evidence of auricular activity is missing, sinoauricular block may be assumed to exist in these tracings. In many tracings there was no standstill of the heart

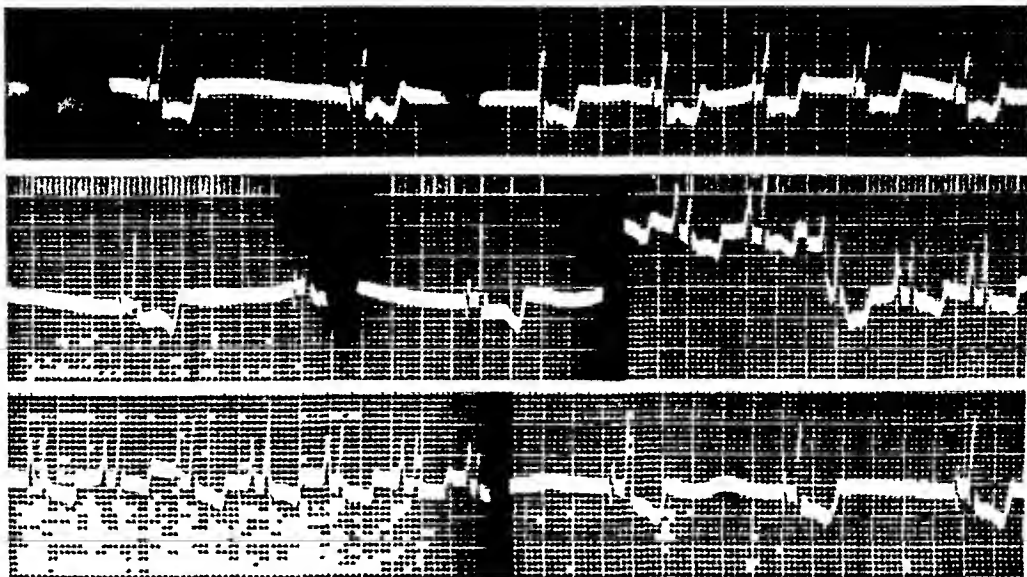


Fig 4a b and c Following repeated injection of a 2% solution of sodium chloride over different portions of the sinus node the same type of sinoauricular block appears, severing of both vagi does not change the result

as in Fig 1 and 2 but an atrioventricular nodal rhythm appeared as in Fig 3. In some of these cases the inverted P-waves preceded the ventricular complexes at a normal distance (coronary sinus rhythm) in other cases P-waves were not visible and were probably buried in the QRS-complexes in rare cases they appeared even after the QRS-complex but before the T-wave (lower auriculoventricular rhythm). Interference between sinus and auriculoventricular rhythm was also seen.

Whenever the sinus node centers are inhibited the 'secondary' centers in the auriculoventricular node perform the duty of pacemaker of the heart. The automaticity of these centers is only slightly less developed than that of the sinus node centers and often it is equally high. Therefore it has been repeatedly pointed out that in order to obtain tracings with bradycardia or long standstill as in Fig 1 and 2, not only is a damage of the sinus node necessary, but also an additional depression of the centers of the auriculoventricular node.¹⁷

In 47 experiments only 9 instances of

sinoauricular block like Fig 1 and 2 were observed. In 14 other instances only deeper centers of the auriculoventricular node became active as in Fig 3. In several experiments repetition of the same injection or injection of other substances produced the same results as the first injection.

Severing of both vagi in the neck did not influence the results.

In the experiment in which Fig 4 was obtained, subepicardial injection of a 2% solution of sodium chloride over the head of the sinus node led to a change of rhythm. The first beat in Fig 4a is an automatic 'escape beat'. The second is preceded by a P-wave of abnormal appearance and is probably conducted from some lower area of the sinus node. After 1.33 seconds (2×0.66 second) another sinus beat appears. The following sinus beat comes after a pause of 1.95 seconds (3×0.65 second). This long pause is also shortened by an escaped beat from a deeper center. The length of the 3 following sinus periods measures 0.66, 0.65, and 0.65 second. The longer pauses therefore closely approximate double and triple the length of one normal period.

Fig 4 was obtained from the same animal

¹⁷ Exster, J. A. E., and Meek, W. J. *Arch. Int. Med.* 1917, 19, 117.

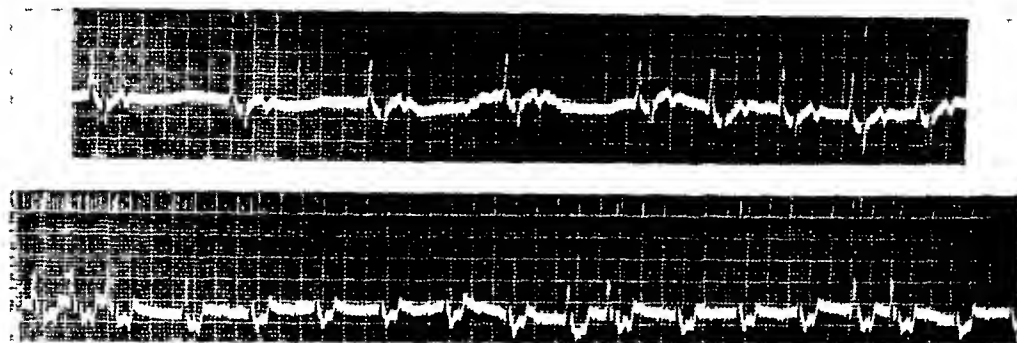


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Respiration in *Macaca mulatta* (Rhesus Monkey)

LEONARD KAREL* AND RAYMOND E. WESTON†

(Introduced by B. B. Rubenstein)

From the Toxicity Section, Medical Division, Edgewood Arsenal, Md

During the course of an investigation of the effects of phosgene on the respiratory exchange of normal animals, it was found that the available literature contains no information on the respiratory exchange in the restrained unanesthetized, non-fasting normal *Macaca mulatta* (Rhesus monkey) despite the extensive use of this species in laboratory investigations. To facilitate the evaluation of metabolic, inhalation, and other studies in monkeys and to permit comparison with the data for other species, the average respiratory rate, tidal air, and minute volume were measured in 39 unanesthetized, untrained, non-fasted macaques. Tidal air and minute volume were calculated in terms of both body weight and body surface area, in an effort to determine which of the latter provides the better basis for expressing respiratory data in this species.

Several months after the original investigation had been completed, it was felt that it would be interesting to include with the other data the respiratory exchange, metabolic rate, and coefficient of oxygen utilization in non-basal monkeys. Such determinations were made on the 4 animals remaining in the laboratory colony.

Methods Immediately before the experiment, the unanesthetized, untrained monkey was weighed and restrained in a spread-eagled, supine position on an animal board. Then, to reduce external stimuli, the eyes and ears were carefully bandaged, and the board was raised to the vertical plane to approximate more closely the semi-erect, normal, simian posture. By means of a facepiece, formed with elastic, adhesive bandage, and low resistance respiratory valves,^{1,2} the

exhaled air was directed into a 4.5 liter McKesson Metabolor, giving a continuous record of time, volumes respired, and individual expirations. The carbon dioxide absorbent and the filters were removed from the spirometer to prevent the absorption of carbon dioxide from the exhaled air and to reduce the resistance of the system. Neither the method for restraining the animals nor the facepiece-valve-spirometer system offered any interference to respiration.

On the basis of dentition and body weight,³ the age of the monkeys was estimated to range from 14 to 36 months. All were sacrificed subsequently, and autopsies were performed. Only normal animals are reported in this series. A diet of fruits, purina fox chow, and water was given *ad libitum* prior to the experiment. No sedation of any kind was administered except as noted below. The approximate mean room temperature and relative humidity were 76°F and 47.6%, respectively.

To give an indication of the state of excitement of the animals, pulse rates were determined within 60 seconds after the completion of each respiratory record. During the period of observation, which was from 10 to 28 minutes, the animals were relatively quiescent. Whenever an occasional animal did struggle, the records were discarded and the experiment repeated.

The respiratory and metabolic data on the 4 macaques were obtained by use of an oxygen-filled, calibrated 300 cc spirometer with an absorber for the exhaled CO₂. Oxygen consumption was measured in the usual manner for indirect calorimetry. Inasmuch as at the later date satisfactory elastic, adhesive bandage was not available for the

* Capt, CWS, AUS

† Capt, MC, AUS

¹ Tobias, J. M., and Weston, R. E., in press² Weston, R. E., and Karel, L., in press³ Huitman, C. G., and Straus, W. L., Jr., *The Anatomy of the Rhesus Monkey*, Williams and Wilkins Co., Baltimore, 1933

about 20 minutes later. Again a bradycardia with a period of 1.36 seconds (2×0.68 second) appeared after injection of 0.1 cc of a 2% solution of sodium chloride at the lower end of the sinus node. A few minutes later both vagi were severed in the neck and a regular sinus rhythm was obtained with large positive P-waves and a rate of 125 beats per minute with each period measuring 0.48 second. The injection was then repeated at the same area and the same tracing as before was obtained. A bradycardia appeared (Fig. 4c) with P-waves of the same abnormal appearance as those following the previous injections, and the length of the period is again 1.36 seconds. Between Fig. 4b and Fig. 4c, 9 QRS-complexes were cut out.

Discussion. The tracings described in the preceding section show that focal mechanical stimulation of the tissue in the neighborhood of the sinus node may lead to the appearance of sinoauricular block. It is difficult to assume any other than a reflex mechanism on the sinus node tissue for this disturbance. A reflex vascular spasm of the arteries supplying the sinus node would not lead to the immediate appearance of the changes of rhythm described above, it would not cause their appearance for from one to 3 minutes. It was impossible to decide whether the inhibition concerns stimulus formation or conduction of the stimulus from the sinus node to the auricle.

The same reflex inhibition which leads to sinoauricular block in some instances also depressed the automaticity in the auriculo-ventricular centers so that long cardiac standstill resulted.

It is possible that focal lesions in the sinus node or in the surrounding tissue, for instance an inflammation as in the case of Hume,⁸ cause sinoauricular block by a similar reflex mechanism. The same mechanism may explain cases of rheumatic fever and sinus block. Here also, focal inflammatory lesions frequently occur. Otherwise sinoauricular block could be expected only if the lesion destroyed all but a small area of the sinus node tissue, and all but one of the junctional bridges between the sinus node and the surrounding auricular muscle.

The instances of sinoauricular block following digitalis medication, comprising approximately 50% of the known cases^{9,10} can be explained only with difficulty. The occurrence of focal myocardial lesions following huge doses of digitalis is known, but these lesions have never been seen with certainty in man, even if they do occur, they can be expected only after much larger doses than those which usually suffice to elicit sinoauricular block.

Summary. The subepicardial injection of 0.1 cc of digitalis or of a strophanthin preparation, as well as of other substances, over the sinus area leads to the immediate but transient appearance of sinoauricular block. This phenomenon is attributed to a reflex inhibition of the sinus node centers caused by the mechanical stimulus and not to a specific effect of the injected substances. In some instances the centers of the auriculo-ventricular node are also inhibited.

⁸ Hume, W. E., *Heart*, 1913, 14, 5, 25.

⁹ Brilow, P., *Lancet*, 1927, 1, 65.

¹⁰ Levine, S. A., *Arch. Int. Med.*, 1916, 17, 153.

TABLE II
Respiratory Data on Monkeys Before and During Exposure to a Phosgene Concentration of 135 mg per Liter

Exper conditions	Respiratory rate per min			Average tidal air			Minute volume		
	Mean	σ	e	Mean	σ	e	Mean	σ	e
Normal respiration	cc 37.6	cc 6.6	cc 1.1	cc/kg 10.1	cc/kg 2.8	cc/kg 0.5	cc/kg 383	cc/kg 120	cc/kg 21
Respiration during phosgene exposure	29.0	10.7	1.8	6.8	1.9	0.3	188	72.5	11.9

eter records. These data are summarized in Tables I and III.

Body surface areas in square centimeters were calculated from the formula

$$S = KW^{2/3}$$

where W is the weight in grams and K is a constant which, for monkeys, equals 11.7 with an average deviation of $\pm 5.4\%$.⁴ The arithmetic means, standard deviations, standard errors, and coefficients of variation were determined in the usual manner.⁵

Under the conditions of these experiments, young adult *Macaca mulatta*, weighing 2.63 kg on the average, had a respiratory rate of 37.6 per minute (coefficient of variation $\pm 17.4\%$), a mean tidal air of 26.8 cc (c v $\pm 32.5\%$), and a mean minute volume of 1014 cc (c v $\pm 39.7\%$). Expressed in terms of body weight or body surface area, the mean tidal air was 10.1 cc per kilogram (c v $\pm 28.2\%$) or 119.6 cc per square meter (c v $\pm 28.0\%$), and the mean minute volume was 383 cc per kilo (c v $\pm 33.7\%$) or 4532 cc per square meter (c v $\pm 34.3\%$).

Because the calculation in terms of body surface area yielded no smaller coefficient of variation than did the simple expression in terms of body weight, minute volumes were calculated in terms of another unit, the body weight in kilograms raised to the 0.73 power which has been recommended as the basis of reference for metabolic and other data.⁶

⁴ Lee, M. O., and Fox, E. L., *Am J Physiol*, 1933, 106, 91.

⁵ Fisher, R. A., *Statistical Methods for Research Workers*, 8th Ed., G. E. Stechert and Co., New York, 1941.

⁶ Benedict, F. G., *Vital Energetics*, Carnegie Inst. Wash. Publ. No. 503, Wash., 1938.

However, this calculation resulted in the following expression for the minute volume

$$\text{Minute volume in cc} = 499W^{0.73}$$

(standard deviation (σ) ± 173.2 , standard error (e) ± 27.7) with a coefficient of variation of 34.7% which is no smaller than that obtained from the calculations in terms of either the surface area or the body weight to the first power.

The average pulse observed in these unanesthetized, untrained monkeys was 215 per minute (c v $\pm 12.7\%$), a value which is apparently too high for the basal state. With the exception of the report by Hartman *et al.*⁷ on fetal and maternal heart rates, there are no data on the pulse rates of unanesthetized, untrained, normal macaques in the available literature for comparison. The rates reported by Hartman *et al.*,⁷ however, agree well with those found for normal non-gravid macaques in the present report. Although it was hoped that the pulse rate would serve as an index of the degree of excitability of the individual animals, no distinct correlation could be made between pulse rate and any of the respiratory data.

A comparison of the data obtained on the normal monkeys prior to and during their exposure to phosgene (Table II) indicates the extent of inhibition of the respiratory exchange during contact with the noxious vapors.² Whereas the uninhibited monkeys had a mean respiratory rate, tidal air, and minute volume of, respectively, 37.6 per minute, 10.1 cc/kg, and 383 cc/kg, the corresponding values during phosgene exposure

⁷ Hartman, C. G., Squier, R. R., and Tinklenburgh, O. L., *Proc. Soc. Exp. Biol. and Med.*, 1920, 31, 28, 285.

TABLE I
Summary of Respiratory Data for *Macaca mulatta*

Sex	Wt kg	Surface m ²	Resp per min	Tidal air			Minute volume			Pulse per min
				cc	cc/kg	cc/m ²	cc	cc/kg	cc/m ²	
M	1.99	185	37	19.4	9.7	104.8	714	359	3790	187
M	2.02	187	29	24.6	12.2	131.2	700	347	3740	208
M	2.03	187	45	19.8	9.8	106.0	886	436	4730	188
M	2.07	190	35	19.0	9.2	100.5	661	319	3512	214
M	2.19	198	34	15.9	7.3	80.6	544	249	2754	210
M	2.19	198	45	21.3	9.7	107.5	948	433	4791	240
F	2.19	198	31	17.7	8.1	89.4	554	253	2805	228
F	2.21	199	41	17.5	7.8	88.2	716	324	3601	164
F	2.24	200	47	19.2	8.6	96.2	901	402	4503	230
F	2.32	205	37	21.6	9.3	105.6	796	343	3929	246
M	2.35	207	36	26.2	11.2	126.7	931	396	4898	218
M	2.53	218	35	31.0	12.2	142.3	1075	425	4935	198
F	2.53	218	43	24.9	9.9	114.7	1069	422	4910	216
F	2.54	218	40	22.7	8.9	104.6	908	358	4173	190
F	2.55	219	34	22.0	8.6	100.7	752	295	3440	224
F	2.56	219	35	16.3	6.4	74.7	572	223	2612	214
F	2.58	220	38	33.2	12.9	150.9	1277	495	5806	212
F	2.58	220	33	28.7	11.1	130.4	937	363	4258	216
F	2.58	220	41	32.7	12.7	148.9	1328	515	6036	230
F	2.61	222	38	21.3	8.2	96.0	800	307	3600	204
F	2.62	222	27	15.0	5.7	67.5	405	155	1835	156
M	2.64	223	37	34.7	13.2	155.5	1267	480	5668	234
M	2.67	225	44	28.2	10.6	125.2	1244	466	5520	246
F	2.72	228	29	52.4	19.3	229.9	1505	554	5600	254
F	2.73	228	24	29.2	10.7	127.8	693	254	3078	164
M	2.75	230	49	31.2	11.4	135.9	1530	556	6660	234
M	2.75	230	36	32.0	8.4	139.4	1164	424	5062	232
M	2.78	232	43	41.2	14.8	177.7	1768	636	7630	150
M	2.81	233	20	27.7	9.9	119.0	554	198	2381	250
M	2.84	235	43	38.4	13.5	163.9	1670	588	7113	240
M	2.87	236	41	28.2	9.8	119.6	1152	401	4871	274
M	2.89	237	44	39.5	13.7	166.5	1734	600	7400	—
F	2.95	241	33	18.6	6.3	77.2	618	210	2571	216
F	3.10	249	43	15.8	5.1	63.6	678	219	2730	228
M	3.10	249	38	26.4	8.5	106.2	1008	325	4055	226
F	3.18	253	48	41.7	13.1	164.9	2004	630	7023	226
M	3.35	262	32	16.3	4.9	62.3	522	156	2000	214
F	3.54	271	17	35.0	9.9	129.1	1288	364	4742	—
M	3.55	272	46	36.5	10.3	134.4	1686	475	6193	190
Mean	2.63	223	37.6	26.8	10.1	119.6	1014	383	4532	215
Std (m)										
Std	0.41	0.22	6.6	8.7	2.8	33.4	403	129	1554	27
Dev (σ)										
Std	0.07	0.04	1.1	1.4	0.5	5.3	64	21	248.8	4.5
Error (ε)										
Coef of	15.6	10.1	17.4	32.5	28.2	28.0	39.7	33.7	34.3	12.7
V _{tr} (σ/m)										
in %										

facepiece, which must form an absolute seal around the nose to obviate leakage, the monkeys were tracheotomized. Determinations on these animals were made both prior to and subsequent to nembutalization. The tracheotomies were performed under local anesthesia, 1.0 cc of a 2% procaine (with adrenalin) solution being used. The distal end of the glass tracheal cannula consisted of a male

half of a standard taper joint by means of which the animal was connected to the apparatus.^{1,2} Nembutalization was achieved by the intraperitoneal injection of 10 mg per kg of nembutal 20 minutes prior to the metabolism tests.

Results The average values for minute volume, respiratory rate, and tidal air were determined for each animal from the spirom-

TABLE II
Respiratory Data on Monkeys Before and During Exposure to a Phosgene Concentration of 138 mg per Liter

Experimental conditions	Respiratory rate per min			Average tidal air			Minute volume		
	Mean	σ	ϵ	Mean	σ	ϵ	Mean	σ	ϵ
	cc	cc	cc	cc/kg	cc/kg	cc/kg	cc/kg	cc/kg	cc/kg
Normal respiration	37.6	6.6	1.1	10.1	2.8	0.5	383	129	21
Respiration during phosgene exposure	29.0	10.7	1.8	6.8	1.9	0.3	188	72.5	11.9

eter records. These data are summarized in Tables I and III.

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where W is the weight in grams and K is a constant which, for monkeys, equals 11.7 with an average deviation of $\pm 5.4\%$.⁴ The arithmetic means, standard deviations, standard errors, and coefficients of variation were determined in the usual manner.⁵

Under the conditions of these experiments, young adult *Macaca mulatta*, weighing 2.63 kg on the average, had a respiratory rate of 37.6 per minute (coefficient of variation = 17.4%), a mean tidal air of 26.8 cc (c.v. = 32.5%), and a mean minute volume of 1014 cc (c.v. = 39.7%). Expressed in terms of body weight or body surface area, the mean tidal air was 10.1 cc per kilogram (c.v. = 28.2%) or 119.6 cc per square meter (c.v. = 28.0%), and the mean minute volume was 383 cc per kilo (c.v. = 33.7%) or 4532 cc per square meter (c.v. = 34.3%).

Because the calculation in terms of body surface area yielded no smaller coefficient of variation than did the simple expression in terms of body weight, minute volumes were calculated in terms of another unit, the body weight in kilograms raised to the 0.73 power which has been recommended as the basis of reference for metabolic and other data.⁶

However, this calculation resulted in the following expression for the minute volume

$$\text{Minute volume in cc} = 499W^{0.73}$$

(standard deviation (σ) = ± 173.2 , standard error (ϵ) = ± 27.7) with a coefficient of variation of 34.7%, which is no smaller than that obtained from the calculations in terms of either the surface area or the body weight to the first power.

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⁵ Fisher, R. A., *Statistical Methods for Research Workers*, 8th Ed., G. E. Stechert and Co., New York, 1941.

⁶ Benedict, F. G., *Vital Energetics*, Carnegie Inst. Wash. Publ. No. 503, Wash., 1935.

⁷ Hartman, C. G., Squier, R. R., and Tinkle, O. L., *Proc. Soc. Exp. Biol. and Med.*, 1930 31, 28, 285.

TABLE I
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F	2.62	222	27	15.0	5.7	67.5	405	155	1835	156
M	2.64	223	37	34.7	13.2	155.5	1267	480	5668	234
M	2.67	225	44	28.2	10.6	125.2	1244	466	5520	246
F	2.72	228	29	52.4	19.3	229.9	1505	554	5600	254
F	2.73	228	24	29.2	10.7	127.8	693	254	3078	164
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Mean	2.63	223	37.6	26.8	10.1	119.6	1014	383	4532	215
(m)										
Std	0.41	0.22	6.6	8.7	2.8	33.4	403	129	1554	27
Dev (σ)										
Std	0.07	0.04	1.1	1.4	0.5	5.3	64	21	248.8	4.5
Error (ε)										
Coef of	15.6	10.1	17.4	32.5	28.2	28.0	39.7	33.7	34.3	12.7
Var (σ/m)										
in %										

facepiece, which must form an absolute seal around the nose to obviate leakage, the monkeys were tracheotomized. Determinations on these animals were made both prior to and subsequent to nembutalization. The tracheotomies were performed under local anesthesia, 1.0 cc of a 2% procaine (with adrenalin) solution being used. The distal end of the glass, tracheal cannula consisted of a male

half of a standard taper joint by means of which the animal was connected to the apparatus.¹² Nembutalization was achieved by the intraperitoneal injection of 10 mg per kg of nembutal 20 minutes prior to the metabolism tests.

Results The average values for minute volume, respiratory rate, and tidal air were determined for each animal from the spirom-

TABLE IV
Comparison of Basal and Non basal Heat Production of *Macaca mulatta*

Benedict* (basal)		Rickett† (basal)			Karel and Weston‡ (Tracheotomized, non basal)		
Wt	Cals/kg/24 hrs	Wt	Sex	Cals/kg/24 hrs	Wt	Sex	Cals/kg/24 hrs
kg		kg			kg		
3.24	52.2	2.7	M	50.5	3.52	M	79.11
3.56	55.9	2.8	M	52.2	3.56	M	93.90
3.84	44.8	2.9	M	48.9	3.26	F	99.52
3.97	51.9	2.9	M	51.0	3.61	F	70.50
4.04	48.0	3.1	M	43.6			
4.18	47.1	3.1	F	49.1			
4.24	46.7	3.5	F	45.1			
4.34	45.4	3.5	F	54.4			
4.35	50.1	3.6	F	41.3			
4.42	49.1	3.6	F	48.5			
4.50	46.7	3.7	F	48.7			
4.58	38.9						
4.73	51.2						
5.10	44.3						
Mean							
(basal)	48.0	Mean (basal)		48.5	Mean (non basal)		85.76

* Values calculated from plotted points on graph made by Dr. George L. Streeter, Dept. of Embryology, The Johns Hopkins University, Baltimore, Md., on "basal" *Macaca mulatta*. A few of the points represent averages which include experiments made both with and without the use of nembutal.⁶

† Data from Table I.⁸

‡ From Table III.

values than would be expected from Kleiber's formula. Consequently, calculating the minute volume in terms of the basis of reference suggested by the Committee on Animal Nutrition of the National Research Council⁶ for computing metabolism yielded a constant which is 2.35 times as great as the constant suggested by Kleiber, with a wide statistical range. The 39 untrained monkeys in this series were not observed under basal conditions as indicated by the pulse rates, but the unanesthetized macaque, as others have noted,⁶ under the usual laboratory conditions is seldom basal, particularly if subjected to any experimental procedures.

The great variability of the respiratory exchange in unanesthetized macaques is another example of individual variation within a presumably uniform population. The animals in this series did not differ markedly in age, weight, nutritional state, or body surface area, and the conditions of the experiment were well controlled. Nevertheless, the large coefficients of variation reported in this paper indicate the wide deviations from the mean in the respiratory response of the individual animals.

Since the respiratory exchange is a reflection of the metabolic activity of the organism which, in turn, is a function of the body mass, calculation of the tidal air and minute volume in terms of body weight did decrease the coefficient of variation somewhat, as would be expected. That recalculating these data in terms of body surface area or of another exponential function of the body weight, the 0.73 power, did not result in an additional decrease in the coefficients of variation is not surprising. For, as Benedict⁶ has emphasized, it is futile to attempt by mathematical means to achieve a uniform expression of even basal metabolic findings despite the "great weight given to complicated mathematical treatment by various writers and the acceptance of these formulas by less critical authors." When to the differences in individual metabolic rates there are added the many other factors which might produce changes in the respiration of any single laboratory animal, the observed range of individual values is to be expected.

It will be noted that the respiratory exchange, particularly in animals No. 3 and 4, of the tracheotomized series was appreciably greater than that found in the series of 39

TABLE III

Respiratory Exchange, Metabolic Rate and Coefficient of Oxygen Utilization in 4 Tracheotomized, Non-basal *Macaca mulatta* Before and After Intraperitoneal Administration of 10 mg of Nembutal/kg

No	Wt	Sex	Pulse before nemb	Tidal air		Resp/min		Min vol		Cals/kg/24 hrs		Cals/sq m/24 hrs		Coef of O ₂ util	
				Before	After	Before	After	Before	After	Before	After	Before	After	Before	After
1	3.26	F	222	15.9	14.5	31	35	493.1	508.0	99.52	90.67	1262.44	1150.07	3.52	3.85
2	3.52	M	240	19.5	11.4	43	39	538.8	442.9	79.11	90.55	1027.61	1176.12	2.35	3.27
3	3.56	M	260	21.0	21.7	48	46	1006.1	998.5	116.25	116.25	1224.47	1615.89	1.53	1.91
4	3.61	F	228	19.8	18.5	50	42	988.2	774.9	70.50	73.61	1031.62	966.24	1.31	1.56
Mean	3.49			17.3	16.5	43	41	756.6	681.1	85.76	92.77	1137.29	1202.08	2.10	2.40

were 29.8 per minute, 6.8 cc/kg, and 188 cc/kg. Statistically, these differences are highly significant.

The complete protocols for the experiment on the tracheotomized macaques appear in Table III. For the non-nembutalized monkeys, the mean heat production was 85.76 Cals/kg/24 hrs or 1137 Cals/sq M/24 hrs, which is approximately 180% of the basal values reported by Benedict⁶ and Rakieten.⁸ Twenty minutes after 10 mg/kg intraperitoneal doses of nembutal, the mean heat production was 92.77 Cals/kg/24 hrs or 1202.08 Cals/sq M/24 hrs. Previously, Rakieten⁸ had reported a similar increase in one rhesus monkey after amytal.

The mean coefficient of oxygen utilization was 2.10% for the non-nembutalized and 2.40% for the "sedated" monkeys. In Table IV a further comparison is made between values calculated from Benedict,⁶ Rakieten's⁸ data and the data on the 4 tracheotomized macaques.

Discussion. On the basis of studies on the basal metabolic rate, Kleiber⁹ proposed that the minute volume (or tidal air per minute as it is termed in his report) for any resting, fasting laboratory animal may be determined from the formula

$$\text{Minute volume in cc} = 212W^{3/4}$$

where W is the body weight in kilograms. He gave no indication of the statistical range of the values calculated from this formula but, as evidence supporting its application, cited the almost exact identity of the calculated minute volume with that observed in one deeply anesthetized, tracheotomized, 3.5 kg Rhesus monkey in which the observed average minute volume was 157 cc per kilogram. The significance of data obtained on one animal is questionable. Moreover, as Kleiber noted, the animal studied was deeply narcotized, a circumstance which depresses not only the metabolic rate but also the respiratory center.

Actual measurement of minute volumes in 39 unanesthetized, non-fasted, untrained Rhesus monkeys (Table I) revealed much higher

⁸ Rakieten, N., *J. Nutrition*, 1935, 10, 357.

⁹ Personnel of U. S. Navy Med. Res., Unit No. 1, and Kleiber, M., *Science*, 1944, 99, 542.

TABLE IV
Comparison of Basal and Non basal Heat Production of *Macaca mulatta*

Benedict* (basal)		Rickett† (basal)			Karel and Weston‡ (Tracheotomized, non basal)		
Wt	Cals/kg/24 hrs	Wt	Sex	Cals/kg/24 hrs	Wt	Sex	Cals/kg/24 hrs
kg		kg			kg		
3.24	52.2	2.7	M	50.5	3.52	M	79.11
3.56	55.9	2.8	M	52.2	3.56	M	93.90
3.84	44.8	2.9	M	48.9	3.26	F	99.52
3.97	51.9	2.9	M	51.0	3.61	F	70.50
4.04	48.0	3.1	M	43.6			
4.18	47.1	3.1	F	49.1			
4.24	46.7	3.5	F	45.1			
4.34	45.4	3.5	F	54.4			
4.35	50.1	3.6	F	41.3			
4.42	49.1	3.6	F	48.5			
4.50	46.7	3.7	F	48.7			
4.58	38.9						
4.73	51.2						
5.10	44.3						
Mean							
(basal)	48.0	Mean (basal)		48.5	Mean (non basal)		55.76

* Values calculated from plotted points on graph made by Dr George L. Streeter, Dept of Embryology, The Johns Hopkins University, Baltimore, Md, on "basal" *Macaca mulatta*. A few of the points represent averages which include experiments made both with and without the use of nembutal.⁶

† Data from Table I.⁵

‡ From Table III.

values than would be expected from Kleiber's formula. Consequently, calculating the minute volume in terms of the basis of reference suggested by the Committee on Animal Nutrition of the National Research Council⁶ for computing metabolism yielded a constant which is 2.35 times as great as the constant suggested by Kleiber, with a wide statistical range. The 39 untrained monkeys in this series were not observed under basal conditions as indicated by the pulse rates, but the unanesthetized macaque, as others have noted,⁶ under the usual laboratory conditions is seldom basal, particularly if subjected to any experimental procedures.

The great variability of the respiratory exchange in unanesthetized macaques is another example of individual variation within a presumably uniform population. The animals in this series did not differ markedly in age, weight, nutritional state, or body surface area, and the conditions of the experiment were well controlled. Nevertheless, the large coefficients of variation reported in this paper indicate the wide deviations from the mean in the respiratory response of the individual animals.

Since the respiratory exchange is a reflection of the metabolic activity of the organism, which, in turn, is a function of the body mass, calculation of the tidal air and minute volume in terms of body weight did decrease the coefficient of variation somewhat, as would be expected. That recalculating these data in terms of body surface area or of another exponential function of the body weight the 0.73 power, did not result in an additional decrease in the coefficients of variation is not surprising. For, as Benedict⁶ has emphasized, it is futile to attempt by mathematical means to achieve a uniform expression of even basal metabolic findings despite the "great weight given to complicated mathematical treatment by various writers and the acceptance of these formulas by less critical authors." When to the differences in individual metabolic rates there are added the many other factors which might produce changes in the respiration of any single laboratory animal, the observed range of individual values is to be expected.

It will be noted that the respiratory exchange, particularly in animals No. 3 and 4, of the tracheotomized series was appreciably greater than that found in the series of 39

monkeys Since, however, the former group was tracheotomized and immediately prior to the tracheotomy had been injected with procaine plus adrenalin, this may account for the substantial difference

While no positive inferences drawn from the data obtained on the few tracheotomized macaques should be applied to non-tracheotomized animals, it is provocative to note that by application of the coefficient of oxygen utilization (2.10%) to the basal values reported by Benedict⁶ and by Rakieten,⁸ one arrives at a mean ventilation rate which is almost the same as the value of 383 cc per kg per minute found as the mean minute volume in the present series of 39 monkeys

The low coefficient of oxygen utilization is probably the result of the increased minute volume Benedict and Benedict¹⁰ have reported appreciably increased ventilation rates and decreased oxygen absorption in normal subjects doing mental multiplications The coefficient of oxygen utilization calculated from the data of Benedict and Benedict¹⁰ fell as low as 2.6%

Summary 1 The average respiratory rate, tidal air, and minute volume were measured in 39 unanesthetized, untrained, young monkeys (*Macaca mulatta*) with an average weight of 2.63 kg ($\sigma = \pm 0.41$ kg, $\epsilon = \pm 0.07$ kg)

2 Expressing the tidal air or minute volume in terms of body weight in kilograms, body surface area in square meters, or body weight in kilograms raised to the 0.73 power resulted in practically identical coefficients of variation

3 The mean values observed were as follows

a Respiratory rate 37 per minute ($\sigma = \pm 6.6$, $\epsilon = \pm 1.1$)

b Tidal air 10.1 cc per kg ($\sigma = \pm 2.8$ cc, $\epsilon = \pm 0.5$ cc) or 119.6 cc per square meter ($\sigma = \pm 33.4$ cc, $\epsilon = \pm 5.3$ cc)

c Minute Volume 383 cc per kg ($\sigma = \pm 129$ cc, $\epsilon = \pm 21$ cc) or 4532 cc per square meter ($\sigma = \pm 1554$ cc, $\epsilon = \pm 248.8$ cc) or 498.7 cc per kg body weight raised to the 0.73 power ($\sigma = \pm 173.2$ cc, $\epsilon = \pm 27.7$ cc)

4 No correlation was found between the animals' pulse rate (mean = 215 per minute) ($\sigma = \pm 27$, $\epsilon = \pm 4.5$), which was intended as an index of excitability, and any of the respiratory data

5 The respiratory rate, tidal air, and minute volume were significantly inhibited in monkeys exposed to a phosgene concentration of 1.38 mg/l

6 a The mean heat production determined by the indirect method in 4 unanesthetized, untrained, tracheotomized macaques with an average weight of 3.49 kg was 85.76 Cals/kg/24 hrs and 1137.29 Cals/sq M/24 hrs—approximately 180% of the basal values reported in the literature

b The mean heat production of the same macaques sedated with 10 mg/kg of nembutal intraperitoneally was 92.77 Cals/kg/24 hrs and 1202.08 Cals/sq M/24 hrs

7 The average coefficient of oxygen utilization in these 4 monkeys was 2.10% for the non-sedated and 2.40% for the nembutalized macaques

The authors wish to thank Lt. Donald R. Le Grive for technical assistance in conducting these experiments

¹⁰ Benedict, F. G., and Benedict, C. G. *Carnegie Inst. Wash.* (Nutrition Laboratory), 1933

15303

Relative Growth of the Kidney in Male Rats

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The weights of organs and of body components are usually recorded as percentage of body weight or in some other simple arithmetic ratio to body weight. The cumbersome quality of such presentation is apparent especially when body weight varies over a wide range. The percentage value generally changes during growth but may become identical for widely different body weights. Klatt¹ calling attention to this points out the very advantageous general applicability of the power formula $y = ax^b$ in which y = the body part or organ, x = body weight and a and b are constants. This had been the basis for the work of DuBois and of Lapicque which successfully solved a number of problems concerned with the relative weight of the brain. He also points out that with an entirely different approach the same formula had been applied to surface and energy metabolism. Moulton² who dealt with the interrelation of body weight, body surface, body nitrogen, weight of blood etc., showed that the handling of such data and the evaluation of the 2 constants can without lengthy calculation be carried out very simply and with sufficient accuracy by graphical methods.

Since then the power formula has found wide application in the hands of Huxley,³ Needham,⁴ Brody,⁵ Kleiber and associates,⁶ Adolph⁷ and others in various problems con-

cerned with organ weights, weights of chemical components of the body, linear measurements of body parts, and measures of physiological functions.

This report presents individual kidney weights and body weights of 150 male stock diet rats and mean weights of 15 consecutive groups of 10 animals each. Kidney weight is given as the combined weight of the 2 kidneys.

In Fig 1 the small circles are individual weights and the crosses represent the mean values for groups of 10. Both scales are logarithmic and $y = ax^b$ plots a straight line. It is seen that the relative growth of the kidney may be divided into 3 phases, each represented by a straight line whose slope indicates the relative rate of growth of kidney and body. As shown in Table I the ratio of kidney weight to body weight (expressed as %) definitely increases during the first phase, it approximates constancy during the second phase, during the third phase it definitely decreases. The percentage values of the first phase repeat themselves in the third phase in reverse order. The same in-

TABLE I
Body and Kidney Weight Data Averaged in
Groups of 10

Mean age days	Mean body wt g	Mean wt of 2 kidneys mg	%
0.1	5.45	44.3	812
1.8	6.70	61.8	922
4.8	9.08*	96.6	1063
6.2	11.75	122.4	1041
10.1	17.04	176.7	1036
12.3	23.75	244.3	1028
17.4	35.72	397.1	1111
27.0	49.54*	555.1	1120
33.2	70.08	744.0	1061
42.7	110.1	1060	963
63.6	162.9	1402	860
79.1	195.7	1694	865
89.4	250.4	1944	776
157	303.4	2379	784
281	408.7	2867	701

* Body weights at which the slope changes of Fig 1 occur

¹ Klatt B. *Biol Zentr* 1919, **39**, 406.
² Moulton C R, *J Biol Chem* 1916, **24**, 299.

³ Huxley J S, *Problems in Relative Growth*, London 1932.

⁴ Needham J, *Biochemistry and Morphogenesis*, Cambridge 1942 p 532 ff.

⁵ Brody S, Davis, H P, and Ragsdale, A C, *Mo Mar Exp Sta Res Bull*, 1937, No 262.

⁶ Weymouth F W, Field, J, and Kleiber, M, *Proc Soc Exp Biol and Med*, 1942 **49**, 367.

⁷ Adolph E A, *Physiological Regulations*, Lancaster 1943.

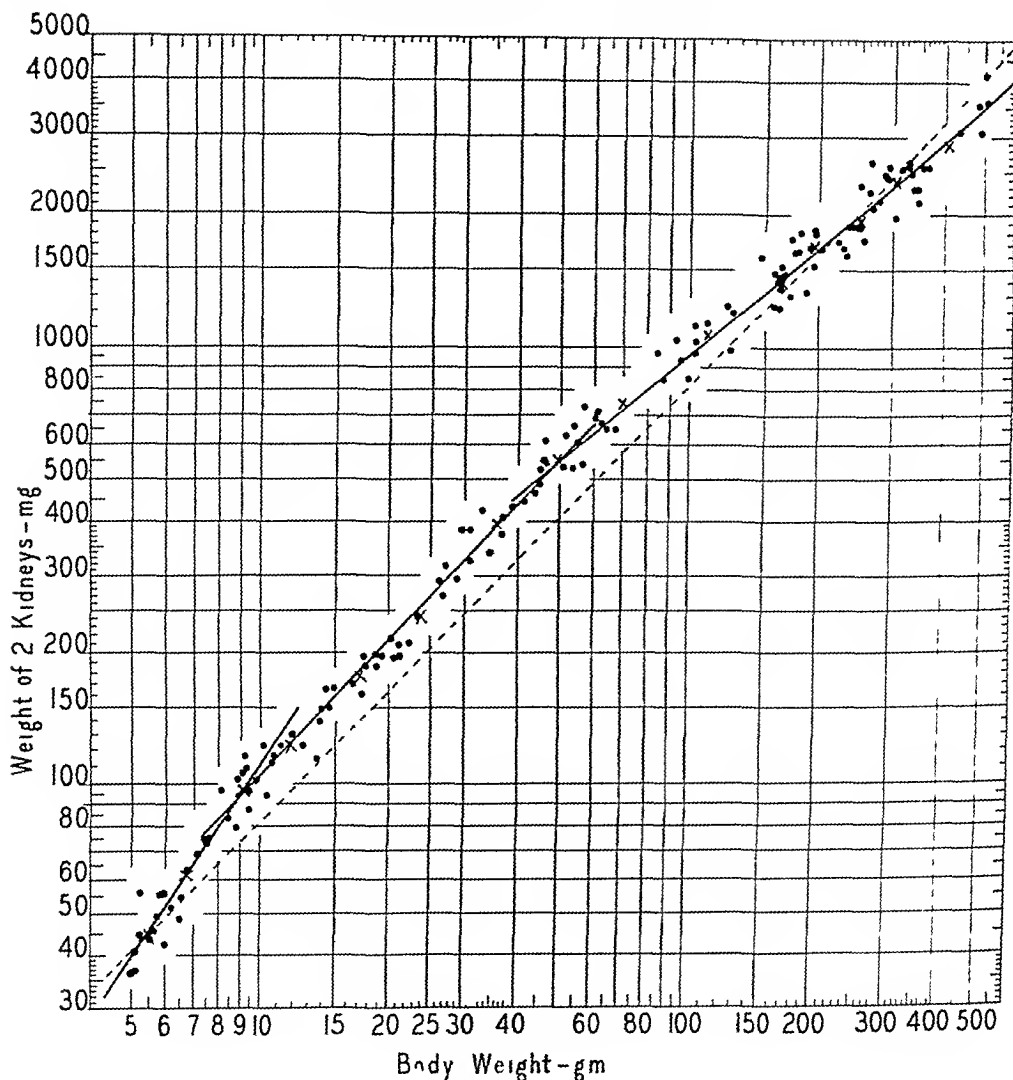


Fig 1

The weight of the kidneys plotted against body weight. Both axes are logarithmic. The points represent individual male rats, the crosses are the average values for 10 successive animals. The slope values indicate that during the first phase the percentage growth rate of the kidneys is 1.44 times that of the body, for the second and third phases the figures are respectively 1.02 and 0.81.

The dotted line (slope 1) indicates the course of relative growth if kidney and body weight remained proportional.

formation can be read from the graph if one considers that constancy of percentage value is represented graphically by a line of 45° to both horizontal and vertical axes such as the dotted line which represents 0.8%. The line of phase 2 approximates 1%.

When taken over the whole of postnatal growth organ weights usually show one or

more breaks. On the other hand general body measures of the rat such as length or surface follow one continuous line throughout. The data of Carman and Mitchell⁸ for instance show excellent adherence to a line with a slope of $\frac{1}{3}$ for body length and

⁸ Carman, G. G., and Mitchell, H. H., *Am J Physiol*, 1926, 76, 380.

a slope of $\frac{2}{3}$ for body surface

Summary The growth in weight of the rat's kidneys in relation to body weight can

be divided into 3 phases. Kidney weight expressed as % of body weight changes continuously but at 3 different rates

15304

Kidney Hypertrophy in B Complex Deficiency

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During a recent series of observations on gastric lesions in B-complex deficient rats our attention was attracted to the kidneys by the occurrence of an advanced case of hydronephrosis. Kidneys in the remaining rats of the group were inspected and weighed. The hydronephrosis may have been purely a chance phenomenon since no more cases were noted. However all the other kidneys, though not abnormal in appearance, were considerably overweight. This seemed to be a point of some interest, especially since inanition controls showed no such enlargement.

The observations are presented individually in Fig. 1 in the form of a log-log plot of the weight of the 2 kidneys against the greatest body weight attained during the experimental period. The straight line represents the course of this relation for stock animals.¹ The B-deficient rats were placed at weaning (28 days, mean weight 64 g) on an ad libitum intake of the deficient diet and were weighed twice a week for a 3- to 4-week period. (Not all the rats survived the 4 weeks.) The maximal weight attained was 68 g, final weight 50 g. Three B-deficient diets were used: 1049, composed of 12% purified casein (Labco), 4% salts, 0.1% carotene in oil (Smaco), 0.3% cod liver oil, 1.6% refined cottonseed oil, and corn starch to 100%; 1051, the same as 1049 but with crude casein substituted for the purified casein, and 1062, composed of 27% Labco casein, 3.4% salts, 2% celluloflour, 0.2% carotene in oil, 0.25% wheat germ oil, 1.55% refined cottonseed oil, and cerelose to 100%. In all cases the diets contained 1.2% modified

Wesson salt mixture (Ca and P free) 1.5% CaCO_3 and enough KH_2PO_4 to bring the total P of the diet to 0.41%. There appears to be no difference between the results on the 3 diets and it is clear that the kidneys weighed about $1\frac{1}{2}$ times what they would in a normal rat of the same body weight as that maximally attained by the B-deficient rats. The difference is highly significant statistically. If comparison were made with the final body weight the difference would of course be even greater.

The inanition controls were of 3 kinds. Control group 1 (partial inanition) was maintained at constant body weight for a 4-week period by limiting the intake of a normal diet. The actual mean weights were: initial 64, maximal 67, final 65. The diet was 1062, supplemented with B factors (1 mg each of thiamine and pyridoxin, 2 mg riboflavin, 4 mg each of calcium pantothenate and nicotinic acid, 200 mg choline per 100 g). Their kidneys appeared to be normal for body weight.

Control group 2 illustrates the effect of a continuous rapid weight loss. Stock rats (40 days, 118 g) in complete inanition, died in about 3 days after losing approximately 40% of their body weight. As the graph shows, their kidneys (represented by crosses) are 20% below the line. Since in these rats the maximal weight is the same as the initial (normal) weight, positions on the normal line in the region of A represent the kidney weights at the beginning of the experiment compared with initial weight; there has been an actual loss of 20% of kidney substance. The final body weights of these rats averaged 73 g; normal rats of this body weight have

¹ Stoerk, H. C., and Zucker, T. F., *Proc. Soc. Exp. Biol. and Med.*, 1946, 61.

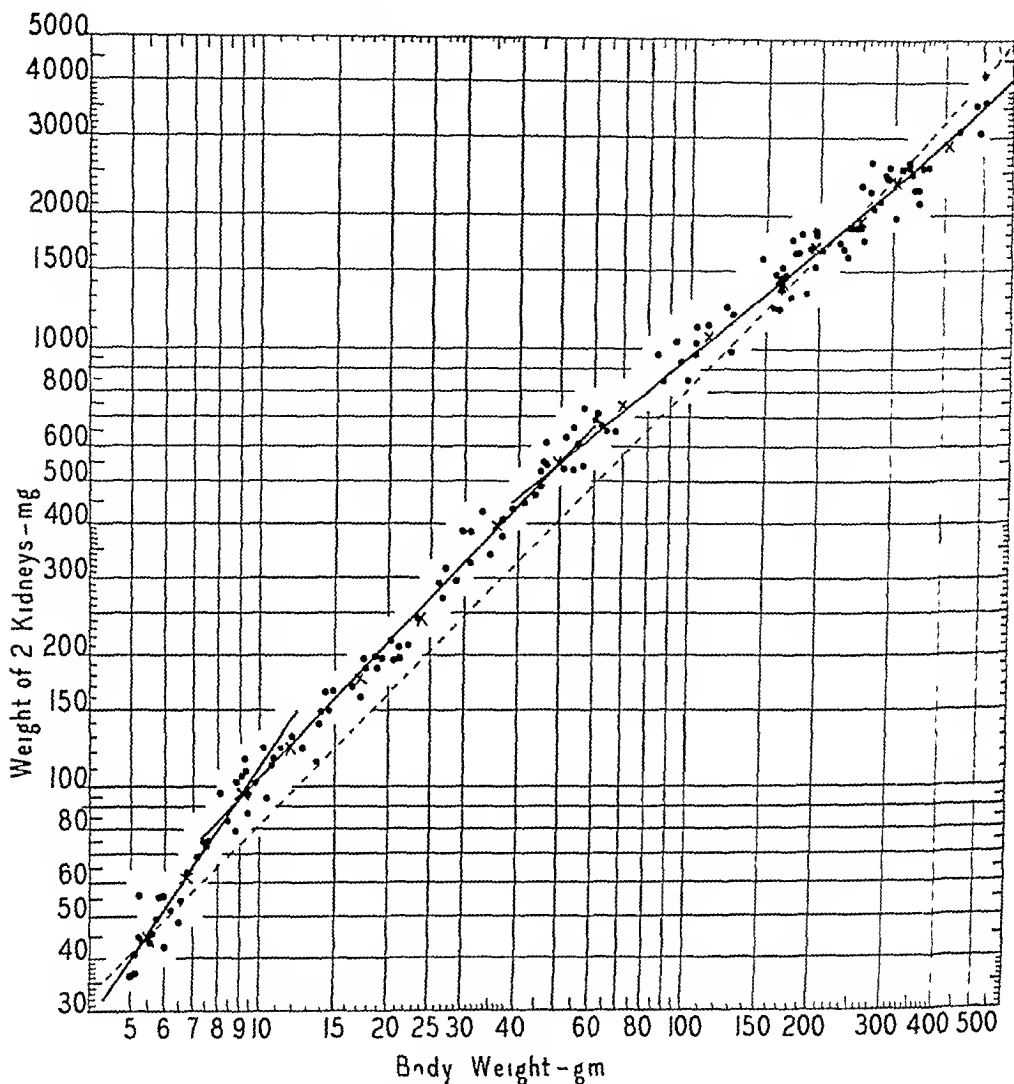


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⁸ Carman, G. G., and Mitchell, H. H., *Am. J. Physiol.*, 1926, **70**, 380.

15305

Observations on Rats Fed with Yellow A.B

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In a previous communication¹ it was shown that the daily ingestion of large amounts of yellow O B (1-O-tolylazo-2-naphthylamine) fail to produce liver cirrhosis or tumors in rats over a period of 250 days of feeding and observation. This study was extended with Yellow A B (1-phenylazo-2-naphthylamine) because this oil-soluble coal tar dye is also extensively used in this country to color foodstuffs.* Yellow A B is somewhat related in structure to Butter Yellow (*p*-dimethylaminoazobenzene) which is one of the azo dyes which readily produces liver cancer in rats.² Because of this chemical relation experiments were undertaken to determine whether or not the ingestion of large amounts of this coal tar dye over a prolonged period might give rise to liver cancer.

Experimental. Yellow A B (National Aniline and Chemical Company) was dissolved in cotton seed oil in the proportion of 6%. Twenty cc of this solution was mixed with 1000 g of coarsely ground unpolished rice, 1000 g of unpolished rice containing 6% of dried whole milk, and thirdly 1000 g of screened Purina Dog Meal (a complete diet). All these 3 diets were supplemented with a small amount of fresh carrots daily. Unlimited water was allowed. Feeding on the various diets containing Yellow A B was continued for about 500 days; all rats still living were sacrificed and examined. Rats male and female (about 150 g body

weight) used in this study were of Sherman stock.

The results showed that the daily ingestion of large amounts of Yellow A. B. in the above diets (each animal having consumed 8 to 12 mg of the dye daily) failed to produce liver tumors or cirrhosis in rats during 50 to 528 days feeding.

Nutrition of young adult rats during ingestion of the Yellow A B-rice diet, Yellow A B-rice and Klim diet and the Yellow A B-normal diet may be briefly summarized as follows.

With Yellow A B-rice diet animals continuously lost weight until death occurred. Of 42 rats 2 died during the first 50 days, 18 died between 51 and 100 days, 12 died between 101 and 150 days and remaining 10 died between 151 and 287 days. The addition of 6% of dried whole milk to Yellow A B-rice diet resulted in a distinct improvement in the health of rats but all of 20 rats died between 70 and 300 days. With the Yellow A B-normal diet animals grew almost normally and were of general good appearance. Of the 22 rats on this last diet 2 died between 50 and 100 days, 6 died between 101 and 150 days, 7 died between 300 and 502 days and 7 were sacrificed on 528 days.

The livers of animals fed the Yellow A B-rice diet with or without supplement of dried whole milk were pale and reddish or yellowish in color, while the livers of animals fed the Yellow A B-normal diet had normal color. There was no great change in the size or shape of the liver, which had smooth surfaces and histological examination revealed no evidence of tumors, bile duct changes or abnormal regeneration of the ducts or liver cells. However some sections (the livers of 8 out of 54 rats that lived more than 100 days) showed focal necrosis of the liver but this was not apparently related to any

¹ Sugita, K. *Proc. Soc. Exp. Biol. and Med.*, 1942, 50, 214.

* According to Mr. W. C. Brambridge of H. Kolbmann Company, New York, the annual consumption of these dyes in U. S. A. is about 44,000 lbs. Therefore, although the results of the experiments with these substances are negative, they are recorded because of general public interest in food dyes.

² Sugita, K., and Rhoads, C. P., *Cancer Research*, 1941, 1, 3.

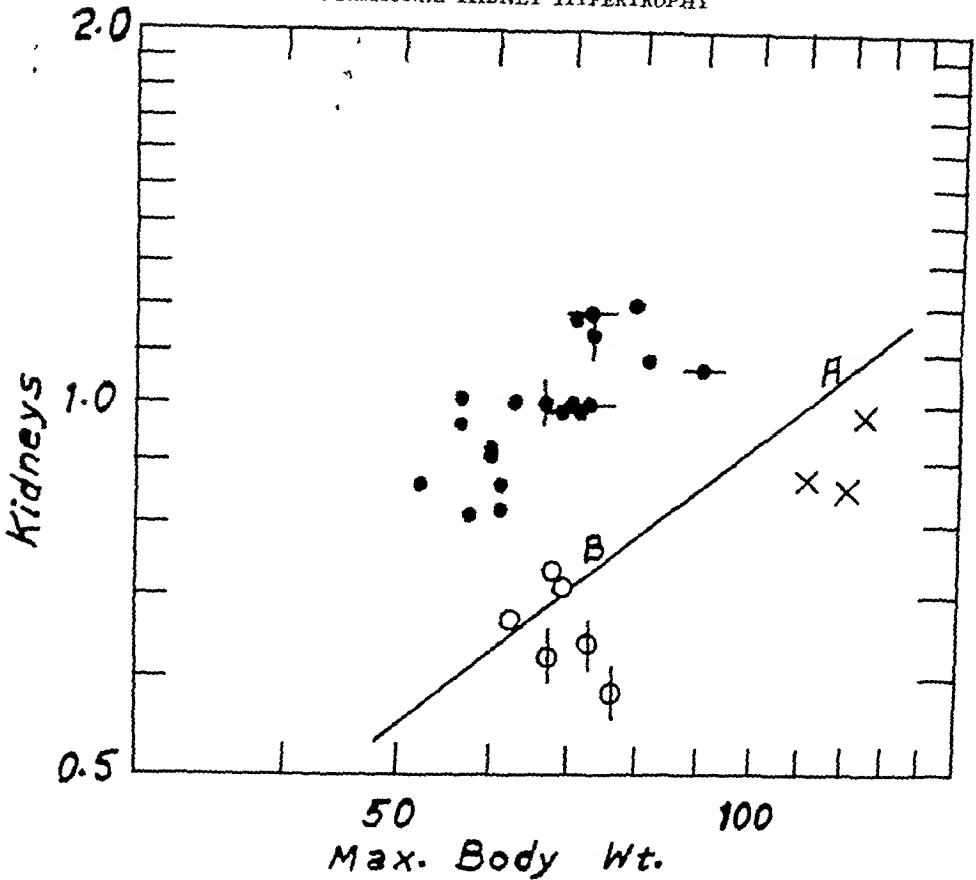


Fig 1

The scales are logarithmic. The line is the position of stock rats. A and B are regions of the graph discussed in the text.

The filled circles represent B complex deficient rats (experimental), the plain ones had 12% vitamin free casein, those with horizontal bars had 12% crude casein, and those with vertical bars had 27% vitamin free casein.

The open circles represent rats on a restricted intake of a normal diet, the plain ones were given enough to maintain weight for 4 weeks (control group 1), and those with vertical bars (control group 3) were the food intake controls on the B deficient rats represented by filled circles with vertical bars.

The crosses represent 6 week old rats which received no food (control group 2).

kidney weights on the line in the region of B, that is, distinctly smaller than the observed kidney weights. Evidently during rapid continuous weight loss the kidneys lose weight but less rapidly than the body as a whole. These differences, each about 20%, are statistically significant, the standard error of estimate for the normal data in the body weight region 50-150 g, is $\pm 5.8\%$, -5.5% .

The third group were pair-fed controls for the B-deficient rats, each received the same daily intake of the normal diet as its pair-mate on 1062 consumed spontaneously. The mean weights of this group were initial

65, maximal 71, final 42. As with the starved rats, the kidneys are about as much too low for the maximal body weight as they are too high for the final body weight.

Summary B-complex deficiency in 20 young rats resulted in kidneys which were 50% overweight. Nine rats which served as inanition controls showed no enlargement. Among the 3 types of food controls those which lost weight had kidney weights below the norm, where body weight was kept constant the kidney weights coincided with the norm based on stock diet animals.

TABLE I
Controls and Summarized Data of Summer and Autumn Series Effect of Choline on Blood Constituents and Cholesterol in Tissues

	NPN mg/100 ml	Urea N mg/100 ml	Glucose mg/100 ml	Serum protein mg/100 ml
Controls 26 (A) s d *	36.2 ± 6.4	19.0 ± 3.0	172 ± 48.4	4.9 ± 0.55
Controls simultaneous 6 (B) s d	34	17.9	222	5.3
Choline (S & A) † 20 (C) s d	37.4 ± 6.0	18.1 ± 8.5	206 ± 8.6	5.7 ± 1.3

Blood	Cholesterol mg/100 ml	Total esters mg/100 ml	Tissue cholesterol		
			Aorta mg/250 mg	Heart mg/250 mg	Liver mg/250 mg
Control 26 (A) s d	248 ± 37		230 ± 43		
Control 6 (B) s d	232.3 ± 20.9	153 ± 5.6	249 ± 29.4	271.3 ± 49.7	337 ± 54.1
Choline (S & A) 20 (C) s d	174 ± 25.4	121 ± 10.8	190 ± 29.2	205 ± 29.3	236 ± 92.9

* s d—Standard deviation

† S & A—Summer and autumn series

TABLE II
Controls and Summarized Data of Winter and Spring Series Effect of Choline on Cholesterol Esters and Phospholipids in Blood and in Tissues

	Cholesterol mg/100 ml		Phospho lipids mg/100 ml	Cholesterol total/esters mg/250 mg		
	Total	Esters		Aorta	Heart	Liver
Controls original old hens (A) 26 s d *	248 ± 37			230 ± 43.2		
Controls simultaneous (B) 6 s d	232 ± 21	153 ± 5.6		249 ± 29	271 ± 49	337 ± 54
Control simultaneous (C) 26 s d	271 ± 37	179 ± 19.2	9.6 ± 2.3	(4) e 230/176 ±45/±20	(4) e 288/233 ±80/±19	(4) e 342/240 ±62/±19
Choline (W & S) † (D) 26 (40-64 days)	179 ± 24	144 ± 9.2	10.1 ± 1.8	165/83 ±50/±24	198/147 ±57/±19	249/210 ±67/±25.6

* s d—Standard deviation.

† (W & S)—Winter and spring series

trols The blood and tissue chemistry of 20 showed definitely lowered total cholesterol and cholesterol ester values in blood and

aorta compared with normal levels (Table I)

The winter-spring (W&S) choline-feeding series of 26 old hens were bled before choline

one diet. The only visceral changes observed were those accompanying the terminal bronchopneumonia. No tumor was found in the visceral organs or elsewhere.

Conclusion. Under the condition of the above experiment, Yellow A B (1-phenyl-

azo-2-naphthylamine) is not a carcinogenic substance.

The author wishes to express his appreciation to Dr. C. P. Rhoads for his interest and valuable advice.

15306 P

Effect of Choline on Blood and Tissues with Especial Reference to Cholesterol in Old Hens *

GEORGE R. HERRMANN (With technical assistance of Anna H. Williams, Mae S. Cox, Lucy Prosise, John Prewett, H. Tom Leigh, and Truett Boles)

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The lipotropic activity of choline in experimental fatty cirrhosis of the liver, has been demonstrated by several groups of investigators.^{1,2,3} The possibility of similar action on other types of cholesterol deposition in other tissue, particularly in atheromatous lesions in the aorta, has suggested itself. The naturally occurring human-like subintimal aortic atheromatosis as reported by Dauber^{4a} was chemically studied in 3-year-old certified 260 to 320 egg RPO sired hens and the average level of certain chemical components established.^{4b} The effect of choline on this pathological chemistry presented itself as an important problem.

Steiner⁵ first and then Bauman and Rusch⁶ and about simultaneously Himsworth⁷

reported that they had not been able to demonstrate any action of choline on the high blood, aortic or liver content of cholesterol in cholesterol-fed rabbits. Andrews and Broun⁸ concluded that choline protected against atherosclerosis and Huber, Broun, and Casey⁹ using lipocaine reported the prevention of atherosclerosis in cholesterol-fed rabbits. Steiner¹⁰ intimated that choline may hasten reabsorption of atheromas.

The data from the original control series A of 26 3-year-old hens are set down for comparison.

A summer-autumn (S&A) and a winter-spring (W&S) choline fed series, each of 26 3-year-old hens individually caged and fed all that they would eat of a high fat and protein laying mash (Purina) and given, in addition, 0.5 g choline chloride daily. With the summer and autumn (S&A) choline series, a simultaneous control series B of 2 immediately sacrificed controls and 4 others that were sacrificed after 14 and 21 days, of the choline-fed series S&A, 6 were after 28, 4 after 35, 4 after 42, 2 after 56, 3 after 70, and 1 after 77 days of choline feeding. There were 6 complete simultaneous con-

*Supported by a grant from the Medical Research Department, Winthrop Chemical Co. Choline chloride was generously supplied by Merck & Co.

¹ Best, C. H., and Rideout, J. R., *Am. J. Physiol.*, 1938, **67**, 122.

² Dragstedt, L. R. et al., *Arch. Int. Med.*, 1939, **64**, 1017.

³ McHenry, E. W., and Patterson, J. M. A., *Physiol. Rev.*, 1944, **24**, 128.

^{4a} Dauber, D. V., *Arch. Path.*, 1944, **38**, 46.

^{4b} Herrmann, George R., *Proc. Soc. Exp. Biol. and Med.*, 1946, **60**.

⁵ Steiner, A., *Proc. Soc. Exp. Biol. and Med.*, 1938, **38**, 231.

⁶ Bauman, C. A., and Rusch, H. P., *Proc. Soc. Exp. Biol. and Med.*, 1938, **38**, 647.

⁷ Himsworth, H. P., *Acta Med. Scand. Suppl.*, 1938, **90**, 158.

⁸ Andrews, K. R., and Broun, G. O., *J. Clin. Invest.*, 1940, **19**, 786.

⁹ Huber, M. J., Broun, G. O., and Casey, A. E., *Proc. Soc. Exp. Biol. and Med.*, 1937, **37**, 441.

¹⁰ Steiner, A., *Proc. Soc. Exp. Biol. and Med.*, 1939, **30**, 411.

in obtaining the Japanese and Russian journals in which information on the vertebrate hosts of the Japanese B encephalitis virus has been published. According to those Japanese reports or abstracts available virus may be detected in the blood, saliva, urine and feces of human beings⁵⁻⁷ and the blood of dogs, rats, horses, goats and sparrows,^{5,6,8} even when no manifest disease is present. Russian workers have reported the isolation of virus from the blood of human patients⁹ and birds¹⁰ of endemic areas, and have reported a high incidence of immunity (35%) in normal horses of the affected areas. Shubladze⁹ concluded that the natural reservoir of the virus is birds and horses. Smorodintsev¹⁰ claimed that wild animals such as deer, wolves and birds constituted the most important reservoir.

It is difficult to interpret Russian and Japanese reports on isolation of this virus as they have announced that a virus encephalitis infection of mice is encountered in certain of their mouse colonies.^{11,12} This virus cannot be readily differentiated from Japanese B either clinically, serologically or by animal susceptibility range. Some Japanese and Russian claims of virus isolations are based on the demonstration of virus after 2 to 6 "blind" serial passages of mouse

brain. When such has occurred, the work cannot be accepted as conclusive. Unfortunately experimental details are not available in many instances, in which virus isolation is reported, and we are therefore unable to draw satisfactory conclusions.

Methods and Materials The technics employed in these studies are in general the same as those reported in our previous studies on St. Louis¹³ and Western equine viruses¹⁴ in the blood of experimentally inoculated chickens. The virus employed was the Nakayama strain, obtained from Lt. Col. A. B. Sabin, M.C.A.S. This virus had been thoroughly mouse brain-adapted by repeated passage. This adaptation was probably a disadvantage but no freshly isolated virus strains were available. Our work with domestic viruses had all been with strains recently isolated from mosquitoes.

All the chickens used were Hampshire reds, incubator hatched, and guarded from mosquitoes and other blood-sucking arthropods till used at from 2 to 3 months of age.

Mice used for the detection of virus were either the Webster Swiss, or the Rockefeller Institute albino strain. They were employed at 3 to 4 weeks of age.

Experiment 1 The first experiment was in the nature of a preliminary trial to determine if chickens would develop a viremia following subcutaneous inoculation of a relatively large dose of virus. Four chickens were inoculated subcutaneously with 1.0 cc of a 10^{-2} dilution of mouse brain virus. The results of the chicken serum and organ tests in Exp. 1 are presented in Table I. It will be noted that virus appeared in the serum 24, 48, and 72 or 96 hours following inoculation of the chickens.

The brain from the single mouse which developed encephalitic signs following the inoculation of a 24-hour serum sample from chicken 934 was passed to 3 mice all of which developed a typical encephalitis. In all other instances when virus was detected, the quantity was adequate to kill all 5 mice.

⁵ Mitamura, T., Kitaoka, M., Watanabe, S., Hosoi, T., Tenjin, S., Seki, O., Nagakura, K., Jo, K., and Shimizu, M., *Tr. Soc. path. jap.*, 1939, 29, 92.

⁶ Mitamura, T., Kitaoka, M., Watanabe, Z., and Tenjin, S., Record 13th Meeting United Assn. of Microbiology of Japan, 1939.

⁷ Mitamura, T., Kitaoka, M., and Watanabe, Z., *Toyo J. Med.*, 1939, No. 3143, 1880.

⁸ Kawamura, R., Kodama, M., Ito, T., Yashiki, T., and Kobayashi, Y., *Kitasato Arch. Exp. Med.*, 1936, 13, 281.

⁹ Shubladze, A. L., *J. microbiol. epidemiol. immunobiol.*, 1943, 12, 87, as reviewed by Rosenthal, L., *Am. Rev. Soviet Med.*, 1944, 2, 166.

¹⁰ Smorodintsev, A. A., *J. Microb. epid., immun., Moshva*, 1942, 11, 12, 67.

¹¹ Kawamura, R., Kasahara, S., Miyata, T., Veda, M., and Yamada, R., *Kitasato Arch. Exp. Med.*, 1940, 17, 38.

¹² Smorodintsev, A. A., personal conversation with W. McD. H.

¹³ Hammon, W. McD., Reeves, W. C., and Izumi, E. M., *J. Exp. Med.*, 1946, 83, 175.

¹⁴ Hammon, W. McD., and Reeves, W. C., *J. Exp. Med.*, 1946, 83, 163.

was started as a simultaneous control series C and 4 were sacrificed after 11 to 13 days for simultaneous tissue cholesterol control. Six hens were re-bled and sacrificed after 40 and 43 days, 10 after 47 and 49, 3 after 50 and 55 days, and 3 after 64 days of choline feeding. The data from the choline-fed series (W&S), sacrificed after 40 to 64 days, showed definite lowering of total cholesterol and cholesterol esters in the blood and in the aortae, as well as in the heart muscle and livers, as set down in Table II.

These data strongly suggest that the administration of 0.5 g choline daily to old hens on a purina laying mash diet for 4 to 10

weeks has little or no effect on the NPN, urea N, glucose, or serum protein levels.

It is, however, evident that the blood cholesterol total and ester levels are reduced and the organic phosphorous values slightly increased. The levels of these substances in the aorta, heart muscle, and liver are likewise changed. Cholesterol seemingly is mobilized from the blood and tissues of hens and metabolized under the influence of choline administration.

The generous cooperation of Mr C W Carter of the Texas Agricultural Experimental Station is gratefully acknowledged.

15307

Japanese B Encephalitis Virus in the Blood of Experimentally Inoculated Chickens *

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During the past 3 years studies on the Japanese B virus have been undertaken in this laboratory with 2 possibilities in mind, first, that United States combat and occupation troops would be entering epidemic areas and second, that the Japanese B encephalitis virus might be introduced on the North American mainland, particularly in the West. In such an event it would be of inestimable value to have as complete a knowledge as possible of the potential animal reservoirs and vectors.¹ The course of our investiga-

tions was influenced largely by our findings in epidemiological studies of the St Louis and Western equine types of infection.^{1,3}

In a separate paper we have reported studies in which 10 common species of Western North American mosquitoes were tested for their ability to act as vectors of Japanese B encephalitis virus.⁴ Of the 10 species tested 7 were demonstrated to be laboratory vectors, and this included those species already proven to be important vectors of the Western equine and St Louis encephalitis viruses.¹ The present paper reports tests of the ability of the chicken, a vertebrate host of the local endemic arthropod-borne encephalitides,^{1,3} to act as a host for the Japanese B encephalitis virus.

Considerable difficulty has been encountered

This investigation was carried out in collaboration with the Commission on Neurotropic Virus Diseases, Board for the Investigation and Control of Influenza and other Epidemic Diseases in the Army, Preventive Medicine Division, Office of the Surgeon General, U S Army, and under a contract recommended by the Committee on Medical Research between the Office of Scientific Research and Development, and the University of California. Aided by a grant from the National Foundation for Infantile Paralysis.

¹ Hammon, W McD, and Reeves, W C, *J Exp Med*, 1947, 35: 994.

² Hammon, W McD, *Calif and West Med*, 1944, 61, 145.

³ Hammon, W McD, Reeves, W C, and Gray, M, *J P H*, 1943, 33, 201.

⁴ Reeves, W C, and Hammon, W McD, *J Exp Med*, 1946, 83, 185.

TABLE II
Lap 2, 4, 5 Results of Tests for Virus Made on the Sera and Spleens of Chickens Inoculated Subcutaneously with 0.3 cc (100 Mouse LD 50 dilution) of Japanese B Virus

[illegible]

* S = Serum
† F = Fresh
‡ U = Unlimited
§ F₁₀ = Frozen

TABLE I

Exp 1 December 1944 Results of Tests for Virus Made on the Serum of Chickens Inoculated Subcutaneously with 1.0 cc of Japanese B Encephalitis virus, Nishiyama Strain, in a 10-2 Dilution

Chicken	Period after inoculation when bled or autopsied and serum or organs tested for virus			
	Serum 24 hr	Serum 48 hr	Serum 72 or 96 hr†	Spleen and brain* 40 days
933	5/5‡		0/5	0/5
934	1/5		5/5	0/5
931		5/5	0/5	0/5
932		0/5	0/5	0/5

* Spleens and brains from all 4 chickens pooled and one set of 5 mice inoculated

† Due to loss of labels the 72-hour and 96 hour bleedings were confused

‡ Numerator indicates number of mice that died and the denominator the number inoculated

inoculated

After 40 days, all 4 chickens were sacrificed, the spleens and brains were pooled and tested for virus with negative results

Experiment 2 Having demonstrated in Exp 1 that chickens would develop a viremia following subcutaneous inoculation of large amounts of virus, Exp 2 was performed to determine in greater detail the length of time during which virus would persist in the serum and the spleen of chickens inoculated subcutaneously with a small amount of virus. A small amount was used because it was wished to duplicate what might occur naturally from a mosquito bite. Eight chickens were inoculated with 0.3 cc of a 100 LD-50 dilution of virus, as determined by titration intracerebrally in mice (1:9,000,000 dilution of mouse brain). Bleedings were begun 24 hours after inoculation and continued every 12 hours up to 120 hours and every 24 hours from 120 to 192 hours after inoculation. Two chickens were bled at each period. Each serum was tested by intracerebral inoculation of 5 mice (Table II). When only one of the 5 mice succumbed a frozen portion of the same serum was inoculated into 5 other mice for confirmation. If 2 or more of the 5 mice succumbed a frozen portion of the serum was titrated in 10 fold dilutions. The death of a single mouse in a group of 5 inoculated with undiluted serum, we do not accept as proof of the presence of virus. Virus was apparently isolated (at least 2 of 5 mice inoculated died at the proper interval), on at least one occasion from the

undiluted serum of 5 of the 8 inoculated birds. Virus was demonstrated from at least one of the 2 chickens tested at 48, 72, 84, 96, 144 and 168 hours after inoculation. At 144 and 168 hours isolations were made from the serum of both birds tested. As an indication that virus titers were low, it may be observed that in no instance was virus demonstrated (by the death of more than one mouse) in a 1:10 dilution of the serum, nor were all mice killed by the undiluted serum as had occurred regularly in Exp 1. Virus was not isolated from the spleens of any of the chickens in tests made at 120, 144, 168 and 192 hours.

Miscellaneous experiments to demonstrate infectivity of chicken blood A single attempt was made to infect a chicken by the bite of infected mosquitoes (*Culex pipiens* Linn.). This experiment was successfully concluded, virus was demonstrated in the chicken's serum 48 and 96 hours after the bite of 4 of the infected mosquitoes. Five out of 5, and 4 out of 5 inoculated mice succumbed respectively from these sera.

Two other chickens, employed in a mite feeding experiment, were bled following subcutaneous inoculation of 0.2 cc and 1.0 cc respectively, of a 10⁻³ dilution of mouse-brain-virus. The serum of the chicken inoculated with the smaller dose, when taken 48 hours after injection, killed all 5 inoculated mice and the serum of the latter, tested at 48 and 72 hours failed to kill any of the mice.

Two attempts to infect mosquitoes from inoculated chickens were unsuccessful. In one

Materials and Methods The majority of the materials and methods employed in the present work were similar to those which have been described in detail in a recent paper⁶. These included methods of maintaining the tsutsugamushi organisms, of determining infective titer, and of performing complement fixation tests. In addition to the Imphal No 8 and Calcutta strains of *R. orientalis* used previously, the Karp^{2b,7} and Kostival⁸ strains were employed in certain of the current tests. Scrub typhus vaccines were prepared in a manner identical with that described⁶ at length for making "crude antigens. In brief, the method was this: white mice, white rats (weight 125-150 g), or cotton rats were injected intravenously with 0.5, 2.0, or 1.0 cc, respectively, of a 10% suspension of infected yolk sac which contained about 10⁵ MLD of *R. orientalis* on the basis of intraperitoneal titration in mice. Lungs and spleens were harvested during the fourth or fifth day when the animals were moribund or recently dead. After the tissues were ground in a mortar with alundum, 10% suspensions were prepared in physiological saline solution and these were freed of large particles by centrifugation in the horizontal machine at 2,000 rpm for 5 minutes. A portion of the supernatant fluid was immediately titered for infectivity. The remainder was promptly treated with sufficient USP formaldehyde solution and merthiolate to bring the final concentrations to 0.1 and 0.01%, respectively. The formalized suspensions were stored at $\pm 5^{\circ}\text{C}$ for one to 6 weeks and then employed in vaccination experiments. The amounts of complement fixing antigen of scrub typhus in most of the materials used as vaccines were determined by titration. These data are given in Table V of the first paper in this series.⁶

Groups of 24 to 70 mice were vaccinated

⁶ Smadel, J. E., Rights, F. L., and Jackson, E. B. *J. Exp. Med.*, 1946, **83**, 133.

⁷ Bengtson, I. A., *Pub. Health Rep.*, 1945, **60**, 1483.

⁸ Blake, F. G., Meyer, K. F., Sadush, J. F., Jr., Kohls, G. M., and Bell, E. J., *Am. J. Hyg.*, 1945, **41**, 243.

with the materials under investigation. Each animal received 3 intraperitoneal injections of 0.5 cc amounts of vaccine given at 5-day intervals. All mice dying during the 24 days following the first injection were autopsied, none showed evidence of scrub typhus infection. Two weeks after the last injection, treated mice were tested for resistance to infection with the homologous strain of scrub typhus. Such challenge tests were performed in the following manner: Serial 10-fold dilutions of fresh infectious yolk sac were prepared in a mixture containing physiological saline solution and 10% normal horse serum and were kept in an ice bath until the challenge inoculation was completed. Six to 10 vaccinated mice were injected intraperitoneally with 0.2 cc amounts of one of the dilutions of infectious suspension. Depending on the available number of vaccinated mice, the dilutions of yolk sac for challenge were so chosen that they should contain a sufficient number of MLD's of *R. orientalis* to cover all or part of the range from 1 to 10⁷. A group of normal control mice was injected with each dilution of the challenge material immediately after the test animals had received portions of the same suspension. Control mice used in these experiments were of the same strain and weight as the vaccinated mice. Infected mice were observed for 21 days and deaths recorded.

The resistance of treated mice to infection with *R. orientalis* was estimated in the following manner. The 50% end-point method of calculating infective titer was applied to the data obtained when groups of treated and control mice were challenged with serial 10-fold dilutions of infectious material. In those experiments in which some mice survived among the group that received the most concentrated challenge material employed it was assumed, for the purpose of calculating the end-point, that if a still more concentrated inoculum had been used then all of the mice would have succumbed. Thus, in Table I, Exp. 3, Vaccine 35 Spleen, only one of 8 mice died when challenged with a 10⁻⁴ dilution of material, nevertheless, it was assumed that all 8 mice would have died if they had received a 10⁻³ dilution of the same challenge

instance 42 mosquitoes fed and in the other 58. Both chickens had been inoculated 48 hours previously with 1.0 cc of a 10^{-3} dilution of mouse brain virus. Five out of 5 and 2 out of 5 mice, respectively, died from inoculation of serum from these chickens at the time of mosquito feeding.

Conclusions As a result of inoculating Japanese B encephalitis virus subcutaneously in chickens, virus can frequently be detected in the serum 24 hours to 7 days later. Even the subcutaneous inoculation of minute amounts of virus frequently resulted in viremia. In addition, it was demonstrated that following the bite of 4 infected mosquitoes infection with viremia occurred in the one chicken used. In these experiments, when employing a "brain-adapted" strain of virus and 2- to 3-months-old chickens, the titer of

virus in the serum was relatively low. It was lower using this agent, than in similar experiments with strains of St. Louis and Western equine viruses recently isolated from mosquitoes. In 2 test feedings on chickens inoculated with Japanese B virus no mosquitoes became infected, but we feel that no conclusions should be drawn from such a limited test. Virus did not persist in the spleen of inoculated chickens over 8 days nor in the brain over 40 days (not tested earlier). These experiments do not conclusively demonstrate that the chicken can or cannot be a source of Japanese B virus infection for mosquitoes, but indicate that such a possibility exists. We feel that chickens and other birds should be considered potential sources of mosquito infection and that they deserve further study with recently isolated strains of virus.

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Studies on Scrub Typhus. II. Preparation of Formalinized Vaccines from Tissues of Infected Mice and Rats *

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Following a number of unsuccessful attempts to prepare a potent inactive vaccine against scrub typhus (see references cited in ¹) materials which showed promise were obtained in 3 laboratories^{1,2,3} almost simultaneously. The first of these vaccines¹ was

* Most of the experimental work discussed here was done in the Virus Division, First Medical General Laboratory, U. S. Army. It was reported to the Director, U. S. A. Typhus Commission, and certain workers in the field in confidential communications listed in reference ³.

¹ Fulton, F., National Institute for Medical Research, London, 21 June 1944; b. Fulton, F., and Joiner, L., *Lancet*, 1945, 2, 729.

² Plotz, H., Bennett, B. L., Regan, R. L., Bell, E. J., Hamilton, H. L., and Snyder, M. J., Report to the Director, USA Typhus Commission, 23 September 1944; b. Plotz, H., Bennett, B. L., and Regan, R. L., *Proc. Soc. Exp. Biol.*

prepared by an adaptation of the rodent lung method used by Castaneda⁴ and by Durand and Sparrow⁵ for the cultivation of rickettsiae of murine and epidemic typhus, a tissue culture method was used for the second type of vaccine,² and rodent tissues for the third.³ The present report describes hitherto unpublished data on the preparation of a formalinized vaccine from the lungs and spleens of rodents infected by the intravenous route.

AND MED., 1946, 61, 313.

³ Smadel, J. E., Rights, F. L., and Jackson, E. B., confidential reports to the Director, USA Typhus Commission, submitted 2 October 1944 and 19 June 1945, respectively.

⁴ Castaneda, M. R., *Medicine, Mexico*, 1938, 18, 607. *Am. J. Path.*, 1939, 15, 467.

⁵ Durand, P., and Sparrow, H., *Arch. Inst. Pasteur Tunis*, 1940, 29, 1.

sistance induced in mice by the scrub typhus vaccines is evident from the following observations. Mice injected intraperitoneally on 3 occasions with 0.5 cc amounts of "normal tissue vaccines" developed no appreciable resistance to infection by the intraperitoneal route with *R. orientalis*. Normal tissue vaccines consisted of formalinized 10% suspensions of lungs of uninoculated mice or formalinized ether extracted 10% suspensions of yolk sacs of uninoculated 12-day-old embryonated eggs. In one experiment with normal mouse vaccine the titer of a given suspension of infectious material was $10^{-7.0}$ in unvaccinated mice and $10^{-7.3}$ in vaccinated animals, in a similar experiment with normal yolk sac vaccine the values were $10^{-8.2}$ in the control and $10^{-8.0}$ in the vaccinated group. Similar results with normal tissue vaccines have been reported by other workers.¹ Tests on 10 impotent scrub typhus vaccines gave further evidence that the degree of resistance obtained in the experiments summarized in Table I was significant. The immunity index of each of these vaccines was in the neighborhood of 1.0, in other words, such vaccinated mice resisted not more than 10 MLD's of rickettsiae. These 10 impotent vaccines were of 2 types: (1) ether extracted formalinized suspensions of infected yolk sacs, and (2) suspensions of mouse tissue with low infective titers (less than $10^{-7.0}$) before inactivation.

Potent scrub typhus vaccines have been prepared from rodent tissue which were highly infectious. The suspension of pooled lung and spleen used to prepare vaccine AMS 2 listed in Table I titered $10^{-8.0}$. The infective titers of the tissues employed as starting material for the other vaccines mentioned in Table I ranged from $10^{-7.5}$ to $10^{-9.0}$; see Table V of the first article in this series.⁶ As already indicated, the immunity indices obtained for 8 lots of mouse vaccine that had titers of 10^{-5} to 10^{-7} before inactivation were never greater than 1.5 and in most instances the values were about 1.0. Therefore, vaccines with definite immunogenic properties were prepared only from tissues with infective titers in the neighborhood of 10^{-8} .

Attempts to estimate the immunizing capacity of a scrub typhus vaccine on the basis of its content of complement fixing antigen have not been encouraging to date. While potent vaccines contained sufficient amounts of such antigen to give titers of 1/3 to 1/6 (Table V, reference⁶), nevertheless, a number of the unsatisfactory vaccines also contained the antigen in some quantity. Furthermore, the serological titer did not correlate sufficiently closely with the infective titer to provide a means of estimating the latter over the range from $10^{-7.5}$ to $10^{-9.0}$.

An assay for determining the immunizing capacity of a scrub typhus vaccine is a lengthy and expensive procedure. In the course of the present work, certain criteria have been adopted which provide a series of checks on a vaccine during its preparation. Since these criteria have been employed, final assay has been avoided on a number of vaccines which would probably have given immunity indices of less than 3.0 and, more important, good vaccines have been produced consistently. The following points are now observed. A strain of *R. orientalis* is not employed for the preparation of white rat vaccines until it is sufficiently well adapted to growth in eggs to provide, with fair regularity, yolk sacs which are infective for mice at a dilution of 10^{-8} . Fortunately, an estimation of the infective titer of each yolk sac inoculum can be obtained rather rapidly by determining the average day of death of mice injected intravenously with 0.5 cc amounts of a 10% suspension of the egg material (Fig. 1). Therefore, at the time white rats are injected intravenously with an infectious yolk sac suspension a group of 4 to 6 mice is injected by the same route with 0.5 cc amounts of the material. If the rats and mice die during the fourth or fifth day then the lung and spleen tissues of the rats are harvested, pooled and titered intraperitoneally in mice. The suspension of rat tissue is formalinized and stored at 5°C during the 21 days required for titration. If the infective titer is 10^{-8} or greater, the vaccine is considered worthy of a final assay for immunogenic activity. Since this system was instituted, 4 successive vaccines prepared

TABLE I
Resistance of Vaccinated Mice to Scrub Typhus

Exp No	Immunized with vaccine number	Dilution of challenge inoculum								Titer 50% lethal (log)	Immunity index (log)
		10 ²	10 ³	10 ⁴	10 ⁵	10 ⁶	10 ⁷	10 ⁸	10 ⁹		
1 (Imphal)	Untreated					4/5	4/5	5/5	1/5	-8.4	
	Mouse 26				2/5	3/6	1/6	3/6		-6.1	2.3
	Lung Mouse 26 Spleen				1/5	1/6	3/6	1/6		-5.4	3.0
2 (Imphal)	Untreated						4/4	3/3	0/4	-8.5	
	Mouse 27 Lung				4/6	1/5	0/5			-5.4	3.1
3 (Imphal)	Untreated					7/8	7/8	4/8	1/8	-7.9	
	Cotton rat 32				3/7	2/6	1/6	0/6		-5.3	2.4
	Lung Cotton rat 34				2/6	1/6	1/5	1/5		-5.2	2.7
	Lung White rat 35		4/8	2/8	5/9	1/9	3/8	0/8		-4.2	3.7
	Lung White rat 35 Spleen			1/8	2/8	3/8	0/8	0/8		-3.9	4.0
4 (Karp)	Untreated	10/10	10/10	8/10	10/10	10/10	9/10	10/10	3/10	-8.5	
	White rat AMS 2	9/10	6/10	5/10	0/10	3/10	1/10	1/10		-4.0	4.5
	Lung & spleen										

Denominator indicates size of group challenged

Numerator indicates number of mice in each group that died from the challenge inoculation

inoculum. The immunity index value for a vaccine represented the difference between the infective titers determined in control and vaccinated mice and was expressed as a positive logarithm.

Results. The results summarized in Table I indicate that, under the conditions of these experiments, mice develop resistance to infection with *R. orientalis* following vaccination with formalinized suspensions of tissues of mice, white rats, or cotton rats that die from scrub typhus. It is apparent from the data presented that resistance varied among individual mice in a group which received a given vaccine†. For example, in Table I, Exp 1, 3 of the 6 mice immunized with spleen vaccine succumbed when challenged with approximately 25 MLD's of *R. orientalis* while 4 of 5 survived 2500 MLD's. In order to overcome difficulties caused by such individual variation, the number of mice in

each group was increased and the range of dilutions employed in the challenge inoculation was broadened. The type of procedure now employed in this laboratory for assay of vaccines prepared from lung and spleen of infected white rats, as well as from tissue cultures of *R. orientalis*,² is illustrated in Table 1, Exp 4. It will be noted that the immunity index in this experiment was 4.5, in other words, the mice resisted about 32,000 MLD's of rickettsiae. It is also apparent from the tabular data that the maximal extent of the immune response elicited in mice was not determined for certain of the vaccines prepared from mice and cotton rats. This was because the lowest dilution of challenge material employed did not kill the majority of the vaccinated mice which received it. Consequently, even though the values of the immunity indices were less for vaccines prepared from these 2 species than for those derived from white rats, no conclusions are warranted regarding comparative immunogenic properties of vaccines prepared from the 3 species.

The immunological specificity of the re-

† Our observations, like those of others,⁸ indicate that mice which survive inapparent infection with scrub typhus consistently resist intra-peritoneal challenge with large doses of *R. orientalis*.

past few months have consisted of pooled lung and spleen tissue of infected white rats, 30 to 35 cc of vaccine were obtained regularly from each animal.

Fulton and Joyner¹ prepared scrub typhus vaccine from lungs of cotton rats infected by the intranasal route with suspensions of mouse lung rich in *R. orientalis*. The vaccines produced by this method and by ours were comparable immunizing agents. Thus the vaccine used in the experiment summarized in their Table VII has an immunity index of 3.7 according to our manner of expressing immunogenic activity. Certain difficulties encountered in the technic of these authors for preparing scrub typhus vaccine have been adequately emphasized.^{1,2} The method we have used avoided or minimized several of these difficulties. In the first place, bacterial sterility of seed inoculum was no problem when yolk sac material was used. Secondly, the intravenous route of inoculation of dangerous material was relatively safe and simple compared with the intranasal route. Finally, white rats were easier to obtain and handle than cotton rats. These advantages are apparent now. How-

ever, the British workers were the first to report on the preparation of a scrub typhus vaccine with some immunological activity and we agree with their statement that 'in wartime—expense and effort are secondary considerations,'^{1,11}

It is apparent from the accompanying paper by Plotz and his coworkers² that scrub typhus vaccine can be prepared from tissue cultures and that such vaccines are as potent as those made from white rats. It was and still is, our opinion that any one of the 3 methods, i.e., intranasal inoculation of cotton rats, growth in tissue cultures, or intravenous injection of white rats, could be used for the production of appreciable quantities of vaccine but that each would present serious technical difficulties for commercial application. It has been adequately shown that the use of yolk sacs infected with the rickettsiae of epidemic typhus has proved suitable for large scale manufacture of a potent vaccine against this disease. However, the application of these methods to the production of scrub typhus vaccine remains to be accomplished.

Summary Formalinized vaccines prepared from lungs or spleens of white mice, cotton rats, and white rats infected with *R. orientalis* are capable of protecting mice against infection with scrub typhus. White rats are the animals of choice for the preparation of vaccines by the method described.

¹ Buckland, F. E., Dudgeon, A., Edward, D. G. F. F., Henderson Begg, A., MacCallum, F. O., Niven, J. S. F., Rowlands, I. W., Van den Ende, M., Birgmann, H. E., Curtis, E. E., and Shepherd, M. A., *Lancet*, 1945, 2, 734.

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Preparation of an Inactivated Tissue Culture Scrub Typhus Vaccine

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The occurrence of scrub typhus in combat areas stimulated research on the development of a scrub typhus vaccine.¹ Three such vac-

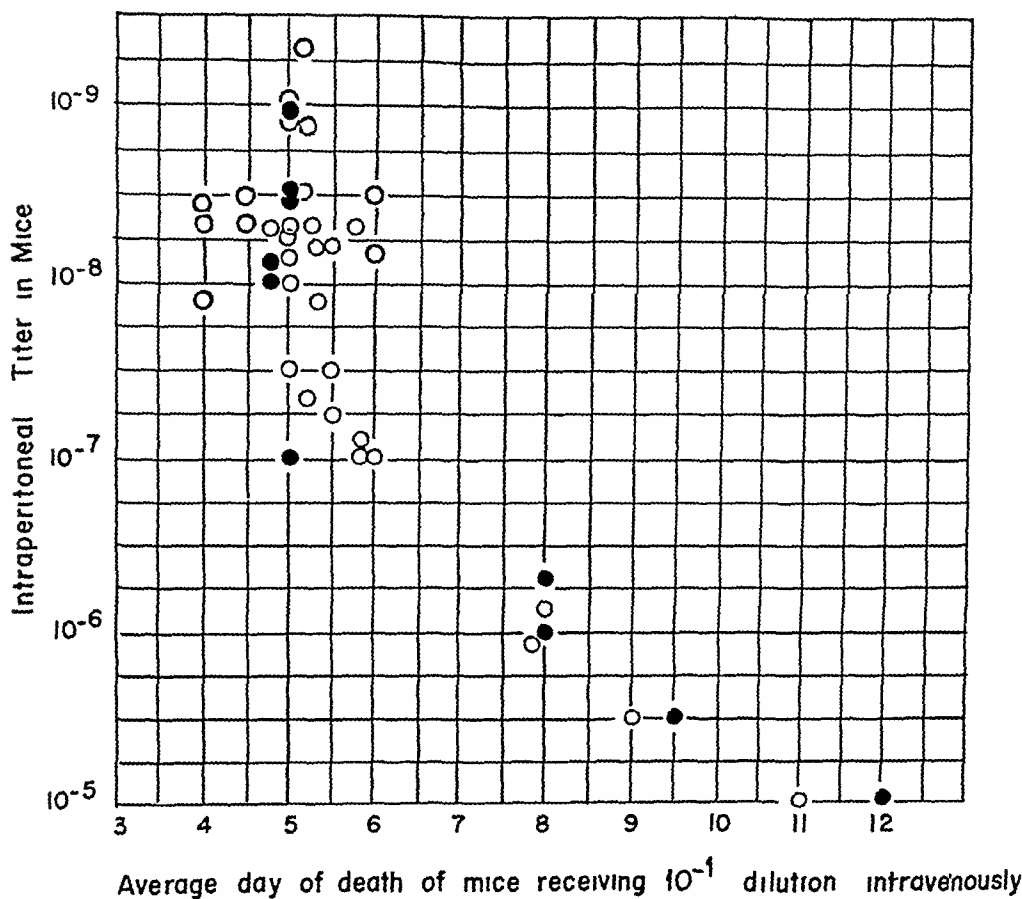
cines have been described. The first of these was prepared from the lungs of intranasally infected cotton rats;² the second from agar

* Member, United States of America Typhus Commission.

¹ Blake, F. G., Marx, K. F., Sidusk, J. F., Jr., Kohls, G. M., and Bell, E. J. *Am. J. Hyg.*, 1945, 41, 243.

² a) Fulton, F., National Institute for Medical Research, London, 21 June 1944; b) Fulton, F., and Joyner, L. *Lancet* 1945, 2, 729; c) Buckland, F. E., Dudgeon, A., Edward, D. G. F. F., Henderson Begg, A., MacCallum, F. O., Niven, J. S. F.,

Fig 1
Correlation of Infective Titer of Scrub Typhus Yolk Sacs with Time of Death of Mice Injected Intravenously with a 10^{-1} Dilution



Open circles in the above graph represent data obtained with 9th to 42nd yolk sac passages of Imphal S strain while solid circles indicate results with 13th to 30th yolk sac passages of the Calcutta strain, respectively.

from white rats infected with Karp or Kostival organisms have been tested and found to have immunity indices of 3.3, 3.3, 4.0, and 4.5. Two batches of vaccine prepared during this period were not assayed because they failed to meet one of the preliminary criteria.

Discussion Although white rats were used in the preparation of the most potent scrub typhus vaccines made during the present studies, infected mice and cotton rats also supplied material for immunologically active vaccines. The white rat offers a number of advantages over the other 2 species of rodents for the preparation of scrub typhus vaccine

on even a laboratory scale, one of the more important is the greater yield of infected tissue. For example, from the 6 white rats used for Vaccine 35, 12.7 g of lung and 7.9 g of spleen were harvested and these tissues had infective titers of $10^{-9.0}$ and $10^{-8.3}$, respectively. In contrast, the 6 cotton rats employed for Vaccine 34, which received the same inoculum used for the rats of Vaccine 35, yielded only 5.7 g of lung and 0.7 g of spleen which had infective titers of $10^{-8.0}$ and $10^{-7.8}$. Thus, with the same expenditure of effort, 20.6 g of starting material was obtained from white rats compared with 6.4 g from cotton rats. Vaccines prepared during the

TABLE I
Resistance of Vaccinated Mice to Scrub Typhus

Vaccine No	Dilution of challenge inoculum									Titer 50% lethal (log)	Immunity index (log)	M L D
	10 ¹	10 ²	10 ³	10 ⁴	10 ⁵	10 ⁶	10 ⁷	10 ⁸	10 ⁹			
440802												
Controls			5/5	5/5	5/6	5/5	2/4	2/6		—7.2		
Vaccine			3/8	0/7	1/7	1/8				—3.0	4.2	15,800
440919												
Controls	10/10	10/10	10/10	9/10	10/10	10/10	10/10	1/9		—7.6		
Vaccine	10/10	6/10	4/10	6/10	3/10	3/10	1/10			—3.9	3.7	5,010
440928												
Controls		10/10	10/10	9/10	10/10	10/10	6/10	1/10		—7.2		
Vaccine		4/8	3/8	1/8	2/8	0/8	3/8			—3.0	4.2	15,800
441003												
Controls	10/10	10/10	10/10	10/10	10/10	10/10	5/10	2/10		—7.2		
Vaccine	6/10	4/10	3/10	2/10	3/10	1/10				—3.2	4	10,000
441009												
Controls	10/10	10/10	10/10	10/10	9/10	8/10	2/10			—7.4		
Vaccine	5/10	8/10	3/10	1/10	2/10	2/10				—3.5	3.9	7,950
441017												
Controls	10/10	10/10	10/10	10/10	10/10	10/10	7/10	9/10		—8.3		
Vaccine	5/6	7/10	2/9	5/10	6/10	2/8				—4.4	3.9	7,950
441024												
Controls		6/6	6/6	6/6	6/6	6/6	3/6	2/6		—7.3		
Vaccine		4/6	2/6	3/6	1/6	0/6	0/6			—2.9	4.4	25,000
110645												
Controls	10/10	10/10	10/10	10/10	10/10	10/10	9/9	7/10	7/9	—9.1		
Vaccine	7/7	9/10	6/10	1/10	2/10	0/10	4/10	0/10		—4.6	4.5	31,600

Denominator indicates size of group challenged

Numerator indicates number of mice in each group that died from the challenge inoculation

flask this solution consisted of one part M/15 Sorenson's phosphate buffer pH 7.0 and 4 parts physiological saline solution, and contained merthiolate or formaldehyde as a preservative. The preservative was used at a final concentration of 1:10,000 of merthiolate or 0.2% of USP formaldehyde, a combination of both was employed in a few instances. The cells were scraped from the agar surface by means of a glass rod and pipetted into a 250 cc Pyrex bottle containing glass beads. The material was maintained at 4°C for 4 to 6 days and then frozen at -20°C overnight. It was then thawed at room temperature and again frozen at -20°C. The next morning it was thawed at room temperature and centrifuged in a horizontal centrifuge at 1,500 rpm for 15 minutes. The supernatant fluid was removed and saved. The sediment, still in the original bottle, was shaken on a machine for one hour. The supernatant fluid which had been saved was added to the shaken material and the mixture was centrifuged in a horizontal machine at 1,000 rpm for 10 minutes. The

supernatant fluid, which represented the vaccine, was stored at +4°C until the results of aerobic and anaerobic cultures and safety tests in mice became available.

The vaccines were tested in the following manner: groups of from 30 to 80 Swiss mice, weighing from 15 to 18 g, were inoculated with the materials under investigation. Each animal received 4 intraperitoneal injections of 0.25 cc amounts of vaccine at 5-day intervals, in some instances 2 intraperitoneal injections of 0.5 cc amounts of vaccine were given at a 7-day interval. Some of the vaccines were also used to immunize mice by the subcutaneous route. Twelve to 14 days following the last dose of vaccine treated mice were tested for resistance to infection with the homologous strain of scrub typhus. For this challenge inoculation serial 10-fold dilutions of fresh infectious yolk sac suspensions were prepared in a mixture containing physiological saline solution and 10% normal rabbit serum and placed in an ice bath until the inoculation was completed. Each mouse received an intraperitoneal injection

tissue cultures,⁷ and the third from the lungs or spleens of intravenously infected white mice, cotton rats or white rats⁴ The present paper describes certain results which have already been reported in a restricted communication³ as well as unpublished data, on the preparation of an inactivated vaccine from agar tissue cultures

Materials and Methods The peritoneal exudate of mice infected with the Kostival¹ or Karp¹ strains were used to initiate yolk sac cultures The early yolk sac passages were rather poor in rickettsiae as judged by stained preparations (fix in methyl alcohol 3 minutes, stain with 2 cc of Giemsa in 58 cc of M/50 buffer solution pH 7.2 for 30 minutes, decolorize with acetone 3 seconds, water) However, by selection and subsequent passages the yolk sacs became richer so that after the 40th passage of the Kostival strain, and the 30th passage of the Karp strain, yolk sacs were occasionally found that appeared as rich in rickettsiae as yolk sacs infected with the Breinl epidemic typhus strain After 55 passages of the Kostival strain about 40% of the yolk sacs were considered rich, this increased to about 80% after 82 passages but no additional improvement occurred up to the present (122 passages) Optimal growth of *R. orientalis* in the yolk sac was found to occur when 5- or 6-day fertile hens' eggs were inoculated and maintained at 35°C for 8 days The embryos usually died on the 10th day under these

conditions The 50th yolk sac passage of the Kostival strain, when titrated intraperitoneally, was infectious for mice at a dilution of 10^{-7} and the 60th passage at 10^{-8} ³ The 36th passage of the Karp strain had an infective titer of 10^{-9} ¹ Yolk sacs that were rich in *R. orientalis* were selected to initiate agar tissue cultures Seed materials from the 50th to 61st passage of the Kostival strain and of the 36th passage of the Karp strain were used in preparing the vaccines mentioned in Table I

The agar tissue culture technic used by Zinsser, Plotz and Enders⁵ for the preparation of an epidemic typhus vaccine was employed in the present studies Ten- or 11-day-old normal chick embryos were removed sterily and after excising the eyes, the embryos were passed through the Fisher press in order to obtain pieces of embryo tissue of approximately the same size The inoculum was prepared as follows infected yolk sac was ground in a mortar with alundum and a 20% suspension was made using the infected yolk fluid as diluent About 7 cc of normal chick cell suspension and 3 cc of yolk sac material were thoroughly mixed, using a 10 cc pipette with a broken end This mixture was placed in the ice box (+4°C) for 20 to 30 minutes and then 2 cc of it were delivered to each flask and evenly spread over the surface of the agar with a bent glass sterile rod After carefully corking the flask with a rubber stopper, the flasks were incubated at 35°C for 8 days At this time the Giemsa stained preparations in a relatively large number of flasks showed the cultures to be rich in intra- and extracellular rickettsiae In contrast to the appearance of the rickettsiae in yolk sac preparations, the organisms in the agar tissue cultures were longer and plumper Infectivity of such cultures for mice was determined on 5 occasions, it varied between 10^{-7} to 10^{-8} ⁶

Vaccines were prepared from tissue culture material in the following manner after discarding the condensation fluid from each flask, 5 cc of a diluent was added to each

Rowlands, I. W., Van den Ende, M., Burgman, H. E., Curtis, E. E., and Shepherd, M. A., *Lancet*, 1945, 2, 734

³ Plotz, H., Bennett, B. L., Reagan, R. L., Bell, E. J., Hamilton, H. L., and Snyder, M. J., Report to the Director, USA Typhus Commission, 23 September 1944

⁴ a Smadel, J. E., Rights, F. L., and Jackson, E. B., Reports to the Director, USA Typhus Commission, submitted on 2 October 1944 and 19 June 1945, respectively, b Smadel, J. E., Rights, F. L., and Jackson, E. B., *J. Exp. Med.*, 1946, 83, 133, c Smadel, J. E., Rights, F. L., and Jackson, E. B. in press

⁷ Isolated by Dr R. Lewthwaite in New Guinea and brought to the United States by the U. S. Navy

⁵ Zinsser, H., Plotz, H., and Enders, J., *Science*, 1940, 91, 51

TABLE II
Effect of Control Materials on Resistance to Scrub Typhus

Vaccine	Dilution of challenge inoculum							Titer 50% lethal (log)	Immunity index (log)	M L D
	10 ⁻²	10 ⁻³	10 ⁻⁴	10 ⁻⁵	10 ⁻⁶	10 ⁻⁷	10 ⁻⁸			
Normal chick tissue "vaccine"	10/10	10/10	10/10	9/10	6/9	9/10		-7 2+	0	0
Epidemic typhus vaccine	9/9	9/10	6/10	7/10	2/10	5/10		-5 4+	1.8	63
Controls	10/10	10/10	10/10	7/9	9/10	6/10	3/10	-7 2+		

TABLE III
Effect of Booster Inoculation on Resistance to Scrub Typhus

Vaccine No. 441206	Dilution of challenge inoculum							Titer 50% lethal (log)	Immunity index (log)	M L D
	10 ⁻²	10 ⁻³	10 ⁻⁴	10 ⁻⁵	10 ⁻⁶	10 ⁻⁷	10 ⁻⁸			
I.P. 2 times	6/7	7/8	5/8	1/8	7/8	0/8		-4.7	1.8	63
I.P. 2 times + booster	6/7	1/7	3/7	1/8	0/8	0/7		-2.8	3.7	5010
Unvaccinated controls	8/8	8/8	8/8	8/8	4/8	3/8	1/8	-6.5		

sistance to infection, the results are summarized in Table III. It is evident that the immunity index obtained when one course of vaccine was given intraperitoneally was 1.8 logs while the index obtained when a booster dose was given was 3.7 logs, almost a 100-fold increase. It is of interest to note that this vaccine when administered subcutaneously, induced no resistance even when the booster technique was employed.

Discussion. The degree of protection afforded by the 3 types of scrub typhus vaccines^{2,3,4} is comparable. The vaccines prepared by Fulton and Joyner² or Smadel, Rights and Jackson⁴ as well as ourselves, elicit immunity when the vaccines are injected intraperitoneally but none when given by the subcutaneous route. It is believed that the intraperitoneal immunity is specific, for control materials inoculated intraperitoneally do not induce such protection. The immunity index induced by the scrub typhus vaccines prepared from the lungs of cotton rats infected by the intranasal route showed an immunity index of 3.7 logs² while

the vaccines prepared from the lungs of white rats, after intravenous inoculation, varied from 2.3 logs to 4.5 logs⁴. Vaccines were prepared from agar tissue cultures which afforded an immunity index of from 3.7 logs to 4.5 logs.

While the manufacture of large amounts of agar tissue culture scrub typhus vaccine would present certain technical difficulties, this method is feasible for making sufficient amounts to protect certain particularly exposed personnel. An advantage in the agar tissue vaccine over the others described is that a relatively clean product can be obtained. Of the various tissue culture methods employed in the preparation of rickettsial vaccines, the yolk sac method no doubt would be the best for large scale production. A successful vaccine has not as yet been prepared from this material, one reason being the deleterious effect of ether upon the antigen.

Summary. Inactivated vaccines prepared from agar tissue cultures infected with *R. orientalis* are capable of protecting mice against infection with scrub typhus.

of 0.2 cc amounts of one of the dilutions of infectious material. An attempt was made to cover the entire range of dilutions from 10^{-2} to 10^{-7} . Normal control mice were inoculated with each dilution of the challenge material immediately after the test animals had received portions of the same suspension. Infected mice were observed for 21 days and deaths recorded. All deaths before the sixth day were considered as non-specific.

The resistance of vaccinated mice to infection with *R. orientalis* was estimated in the following manner. The 50% end-point method (Reed-Muench) of calculating infective titers was applied to the data obtained when groups of vaccinated and control mice were challenged with serial 10-fold dilutions of infectious material. In those experiments in which some mice survived among the group that received the most concentrated challenge material employed, it was assumed, for the purpose of calculating the end-point, that if a still more concentrated inoculum had been used then all of the mice would have succumbed.

Results The results obtained in immunological tests on 8 scrub typhus vaccines are summarized in Table I. It is observed that under the conditions of these experiments, an immunity index of from 3.7 logs to 4.5 logs was found. In other words, these vaccines induced protection against from 5,000 to 32,000 MLD's of *R. orientalis*. A number of other vaccines were prepared which failed to show the relatively high immunity index displayed by those illustrated in Table I. These had indices of 1.1 to 2.43 logs (2.3 - 2.4 - 2.43 - 1.1 - 2.1 - 1.9 - 1.7 logs). It is felt that these poorer vaccines resulted from the fact that the culture flasks were not adequately examined so as to include in the vaccines only those cultures that were rich in *R. orientalis*.

The vaccines mentioned in Table I elicited a demonstrable resistance in mice when they were injected intraperitoneally and the animals were challenged by the same route. When certain of these potent vaccines were administered subcutaneously and the mice were challenged subsequently by the intraperitoneal route, the immunity indices varied

from zero to 1.9 logs.

The immunological specificity of the resistance induced by scrub typhus vaccines was evident from control experiments. A normal chick tissue "vaccine" was prepared from agar tissue cultures in the same manner as was the scrub typhus vaccines except that infectious inoculum was omitted. The mice received 2 intraperitoneal injections of 0.5 cc at 7-day intervals and were challenged after 14 days. No protection was elicited in the treated mice, the infective titer was $10^{-7.2}$ in test and control animals. Likewise, a similar experiment was performed using a commercially prepared epidemic typhus vaccine. The immunity index was 1.8 logs or 63 MLD's protection. These data are summarized in Table II.

A series of experiments in which mice received "booster" injections of vaccine illustrate the value of this type of immunization procedure. A group of mice that had received the usual course of injections of vaccine 441009 was held for 30 days and then given a booster injection of 0.5 cc of the vaccine by the intraperitoneal route. The vaccine had an immunity index of 3.0 logs when tested by the usual method (Table I) but when the booster injection was employed, the index became 4.9 logs. Thus, the induced resistance increased from 7,950 to 80,000 MLD's. A similar set of comparative experiments was made with vaccine 441024. The immunity index was 4.4 logs when the ordinary assay was done but rose to 5.4 logs in the experiment in which the booster injection was used. Here again the protection was increased 10-fold, from 25,000 to 250,000 MLD's.

The salutary effect of booster injections is even better illustrated by the results obtained with a poor scrub typhus vaccine. Vaccine 441206, made with the Kostival strain and inactivated with 0.2% formalin, was injected intraperitoneally or subcutaneously into 2 groups of mice. 0.5 cc doses were given on 2 occasions 7 days apart. The animals were all held for 30 days when half of each group received a second injection of 0.5 cc of the same vaccine by the original route. Twelve days later all the mice were tested for re-

TABLE I
Effect of Exudative Material on Number of Circulating Leukocytes in the Guinea Pig

Guinea pig No	Amt of exudate inj	Basal white blood cell level	Highest level of white blood cells attained within 4 hrs after inj of exudate	Absolute increase in white blood cells
	cc	per mm ³	per mm ³	per mm ³
Z	0.5	6,640	9,500	2,860
11	1	13,500	21,800	8,300
W	2	16,850	19,675	2,825
J	3	3,325	11,500	8,175
24 86	5	15,400	27,500	12,100
V	5	13,000	22,050	9,050
Avg		11,453	18,671	7,218

TABLE II
Effect of the Leukocytosis promoting Factor (LPF), Injected Intraperitoneally, on Leukocyte Level in the Guinea Pig

Guinea pig No	Amt of LPF	Basal WBC level (per mm ³)	Highest level attained within 4 hr after injec (per mm ³)	Absolute increase in WBC (per mm ³)
X	3.0 cc	12,600	17,725	5,125
11	5.0 "	13,200	21,800	8,600
24 39	5.0 "	8,712	12,175	3,463
24 88	5.0 "	15,850	17,375	1,525
100	6.0 mg	4,638	7,150	2,512
9	6.0 "	8,500	15,750	7,250
24 36	8.0 "	3,600	11,275	7,675
17 60	8.5 "	12,750	18,400	5,650
24 87	11.0 "	12,727	30,575	17,848
24-47	12.0 "	9,075	14,550	5,475
11	13.0 "	17,225	58,975	41,750
24 36	22.0 "	7,725	15,625	7,900
Avg		10,550	20,115	9,565

leukocytes. The average increase in the number of circulating leukocytes is 7,218 or 63%. This increment in the white cells of guinea pigs is about of the same magnitude as encountered previously on dogs¹. The actual effect throughout the duration of an experiment is illustrated in Fig 1 (g p 24-86, Table I).

Having established the presence of the leukocytosis-promoting factor in dog exudate, which is apparently active on the number of circulating leukocytes in the guinea pig, observations were undertaken in an endeavor to determine whether the active principle, *i.e.*, the LPF itself is likewise active in the guinea pig. The factor was recovered from exudates, with only slight modifications essentially as described by one of us in an earlier study⁸. The material dissolved in

saline was injected in varying doses (Table II) into the peritoneal cavity of guinea pigs. In 9 out of 13 experiments, the LPF definitely increased the number of circulating leukocytes of the guinea pig. When, as explained above, the disadvantages attending intraperitoneal injections are taken into consideration, it is readily seen that 9 out of 13 experiments in which activity was demonstrated seem to be of definite significance. The average increase in the number of circulating white cells is 9,565 or 90.7%. The recovery of the LPF from the crude exudate, in which other factors are likewise present, readily explains the somewhat more pronounced effect obtained (*cf* Table I and Table II). The course of an experiment with the leukocytosis-promoting factor (guinea pig 24-87) is illustrated in Fig 1. A study was also undertaken to determine the normal range of variation in the white

⁸ Menkin, V., *Arch. Path.*, 1945, 39: 28

A Convenient Test Animal for the Detection of the Leukocytosis-Promoting Factor of Exudates*

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Earlier studies have demonstrated in inflammatory exudates the presence of a factor liberated by injured cells and *per se* capable of reasonably explaining the mechanism of leukocytosis associated with numerous inflammatory processes.¹ This factor has been termed the leukocytosis-promoting factor (abbreviated as LPF). Chemically, the active material is associated with the pseudoglobulin fraction of exudates.^{2,3} Besides being capable of discharging immature leukocytes into the circulating blood, the LPF induces a specific growth effect on granulocytes and megakaryocytes in the bone marrow.⁴ The presence of the factor in the exudates of rabbits has been confirmed by Reifstein.⁵ These studies have also received confirmation in the hands of Page and his associates.⁶

The purpose of the present brief communication is to demonstrate that the guinea pig can also be conveniently utilized as a test animal for the presence of the leukocytosis-promoting factor in canine inflammatory exudates. Prior to this study, observations have been carried out exclusively on dogs. The finding of a smaller test animal for the assay of LPF would obviously be of distinct advantage for future studies.

Experimental Exudative material was

* This represents No. 33 in a series entitled "Studies on Inflammation." This study was aided in part by a grant from the Duke University Research Fund.

1 Menkin, V., *Am. J. Path.*, 1940, **16**, 13.

2 Menkin, V., *Arch. Path.*, 1940, **30**, 363.

3 Menkin, V., and Kadish, M. A., *Am. J. Med. Sci.*, 1943, **205**, 363.

4 Menkin, V., *Am. J. Path.*, 1943, **19**, 1021.

5 Reifstein, G. H., Ferguson, J. H., and Weiskotten, H. G., *Am. J. Path.*, 1941, **17**, 233.

6 Taylor, R. D., and Page, I. H., *Am. J. Med. Sci.*, 1944, **208**, 281.

obtained from the pleural cavity of dogs by the intrapleural injection of turpentine, as previously described.⁷

The whole exudate, in quantities varying from 0.5 to 5 cc., was injected into the peritoneal cavity of guinea pigs following the determination of the basal white cell count. The blood sample was obtained by nicking the toe-pad with the sharp blade of a razor. The exudative material was injected intraperitoneally, for technical convenience, rather than intravascularly, as in previous studies. This method, however, entails certain disadvantages. Injection into the peritoneal cavity may be accompanied by the puncture of a visceral structure with consequent introduction of the material into either intestine or some other organ. Furthermore, white counts were made every hour subsequent to the injection of the exudative material for a period of about 4 hours. It was found that after a longer interval a frank peritonitis frequently developed, which in turn liberated its own LPF and thus confused the results. But by reducing the period of study to the first 4 hours following introduction of the material, this error could to a large extent be eliminated. Nevertheless, intraperitoneal injections obviously favor a less efficient absorption of material than direct intravascular injection. As a consequence, the final results obtained show definitely more individual variation than was encountered in dogs.

The results following intraperitoneal injection of canine exudative material are assembled on Table I. It is clear that at least in 4 out of 6 experiments the introduction of an exudate was followed by a definite increase in the number of circulating

⁷ Menkin, V., *Am. J. Path.*, 1934, **10**, 193.

EFFECT OF LEUKOCYTOSIS-PROMOTING FACTOR ON THE CIRCULATING LEUKOCYTES OF THE GUINEA PIG

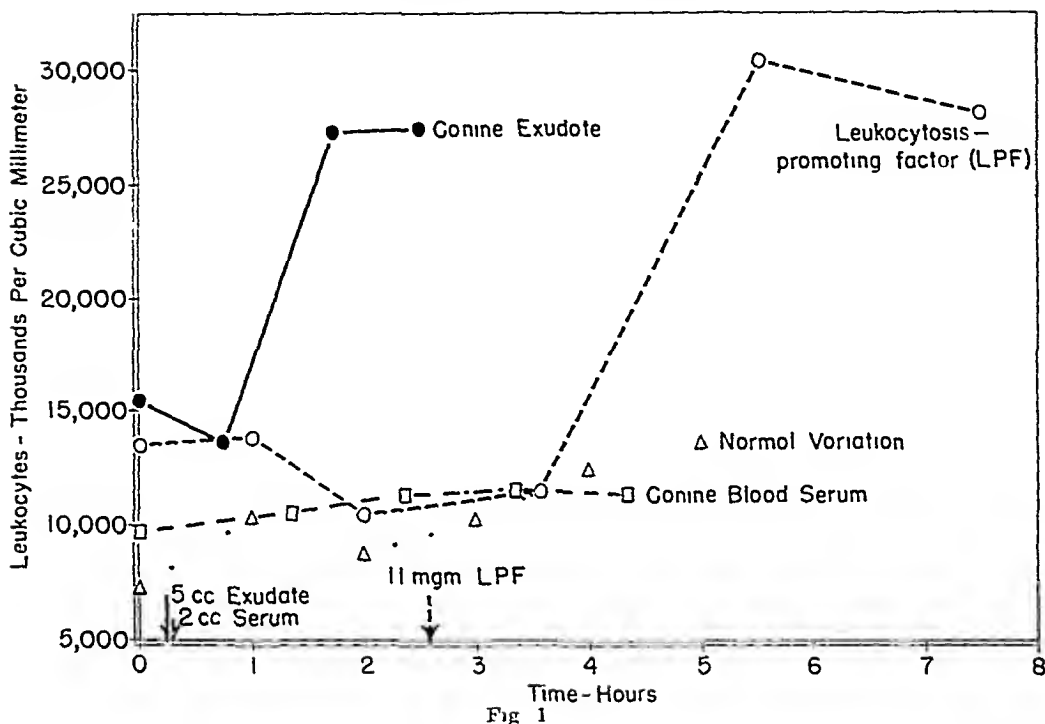


Fig 1

cell count during a period of about 5 hours in uninjected guinea pigs. The data are presented in Table III (Fig 1). Except for one animal in which there was an increase of 6,450 cells, the usual range of maximum rise in white cells definitely tended to be lower than in the injected guinea pigs (Tables I, II and III). The average increase was 3,057 cells or 36.9%. It is quite clear from a survey of observations of this nature in comparison with observations on experimental animals, (Tables I and II) that the leukocytosis-promoting factor obtained from a canine exudate is definitely active in inducing a leukocytosis in the guinea pig when the active substance is injected intraperitoneally.

Nevertheless, in view of the wide variations obtained from animal to animal when intraperitoneal injection of the active substance was employed, and in view, as explained above, of the reason for these fluctuations in results, a different route of

injection was used in a series of experiments. The leukocytosis-promoting factor or LPF either in the fluid state or in dry form and dissolved in 5 to 10 cc of physiological saline was now injected subcutaneously. This was done, as a rule, in the region of the thigh. Such procedure induced a very rapid rise in the number of circulating leukocytes. Sometimes as early as a half hour after introduction of the active material, an appreciable leukocytosis ensued. The period of an experiment extended, as a rule, for about 4 hours. During this interval a leukocytosis usually developed. The rise in the number of circulating leukocytes was found to be appreciable in 11 out of 12 experiments (Table IV). The average rise in 12 experiments was found to be 11,158 or a rise of 72.4%†. The course of one experiment is

† The fact that the average basal level in the series of guinea pigs listed in Table IV is somewhat higher than in the other series of experiments is largely explained by the fact that 2 of

TABLE III
Variation in Number of White Cells in Blood of Normal Guinea Pig

Guinea pig No	Lowest No of leukocytes per mm ³ within about 5 hr	Highest No of leukocytes per mm ³ within about 5 hr	Absolute increase in white blood cells per mm ³
11	16,025	17,950	1,925
24 32	8,525	13,400	4,875
24-96	6,805	9,180	2,375
100	5,050	7,975	2,925
24 39	7,850	9,725	1,875
24 47	7,375	13,825	6,450
24-36	6,300	7,275	975
Avg	8,276	11,333	3,057

TABLE IV
Effect of Leukocytosis promoting Factor (LPF), Injected Subcutaneously, on Leukocyte Level in the Guinea Pig

Guinea pig No	Amt of LPF	Basal WBC level (per mm ³)	Highest level attained within 4 hr after inject (per mm ³)	Absolute increase in WBC (per mm ³)
2 23	40 mg	15,150	23,500	8,350
2 23	35 "	18,150	32,950	14,800
2 24	39 "	19,900	26,900	7,000
2-25	40 "	9,650	15,900	6,250
2 26	5 cc	12,100	22,000	9,900
"	10 "	12,350	22,950	10,600
2 28	10 "	11,950	14,600	2,650
2 31	10 "	12,100	28,450	16,350
2 29	10 "	10,050	23,950	13,900
b	5 "	8,000	18,700	10,700
17 58	42 mg	29,300	53,200	23,900
2 32	35 "	26,200	35,700	9,500
Avg		15,408	26,566	11,158

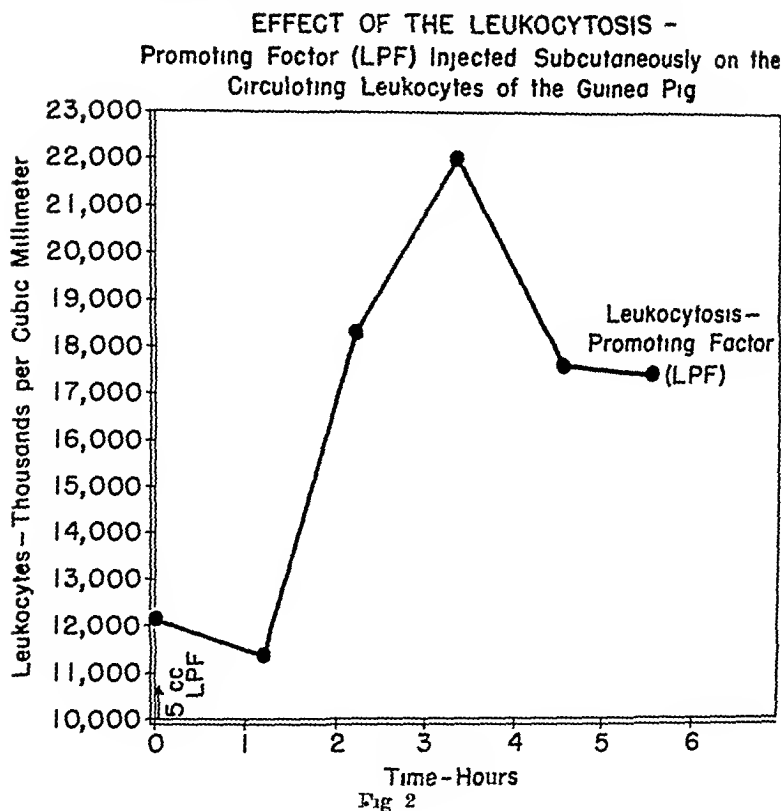
TABLE V
Effect of Injections in Peritoneal Cavity and in Subcutaneous Tissue of the Guinea Pig of Various Materials Other than Exudate or LPF

Guinea pig No	Type and amt of injected material	Basal WBC level (per mm ³)	Highest level attained within 4 hr after inject (per mm ³)	Absolute increase in WBC (per mm ³)
20	Intraperitoneal injection			
	1 liver extract concentrated in barbitalate buffer	10,925	13,925	3,000
A	5 saline	12,850	15,600	2,750
24 36	2 human serum	5,775	8,750	2,975
B	2 canine serum	9,500	11,600	2,100
C	2 " "	11,100	12,700	1,600
D	2 5 broth	10,100	10,800	700
	Subcutaneous injection			
2 33	10 saline	10,550	13,900	3,350
2 34	5 "	9,800	14,350	4,550
2 38	5 "	15,700	20,700	5,000
2 35	5 necrosis	11,850	13,250	1,400
2 36	2 "	10,950	11,000	50
	Avg	10,827	14,325	2,498

exudates, particularly if the material is injected into the subcutaneous tissue

Finally, as pointed out frequently in the past, these studies may have clinical application, for it is well known that the prognosis of an infectious process is to a large extent referable to the number of circulating leukocytes. For this reason studies have been initiated to determine the effectiveness of the leukocytosis-promoting factor on man. These studies are now in progress and will form the subject of a separate future communication *in extenso*. It can be

pointed out, however, that 8 different patients with normal white cell counts have been injected intravenously with doses ranging from about 18 to 230 mg of active canine LPF. Within a few hours the white blood cell count increased without any change in temperature, and within several hours the count had risen 80 to 150%, showing that the material is both innocuous and potent on human beings. These studies are being carried out by 2 of us (V M and E U) in collaboration with Dr E G Goodman of the Department of Medicine.



illustrated in Fig 2. It is thus quite clear that the results are more constant and dependable when, in the guinea pig, the subcutaneous route of infection of the leukocytosis-promoting factor was employed.

Finally, as an additional control, various types of material unrelated to the leukocytosis-promoting factor of exudates have been injected into the peritoneal cavity and the subcutaneous tissue of guinea pigs. Those have included diversified substances such as liver extract, saline, broth, human and canine blood serum, and necrosin recovered in turn from exudates. The results of these experiments are assembled together in Table V (also Fig 1). It is clear that, in contrast to the effect of the exudate and the leukocytosis-promoting factor, these materials have failed to alter appreciably the number of circulating leukocytes. The average maxi-

mum rise in the number of leukocytes is 2,498, or 23%. It is to be noted that these non-specific substances were all injected as solutions, precisely as in the case of the exudative material or the LPF. This is important, for when an insoluble particulate material is injected, for instance, in the peritoneal cavity, a peritonitis is prone to develop early, and this may in turn confuse the final interpretation of results.

Conclusions An exudate obtained from a dog, which contains the leukocytosis-promoting factor, induces a leukocytosis when injected into the peritoneal cavity of a guinea pig. Similar results are obtained with the recovered leukocytosis-promoting factor of exudates injected either intraperitoneally or subcutaneously. The effect is quite specific. It fails to occur when other types of soluble materials are utilized. Consequently, the guinea pig presents itself as a suitable test animal for detecting the presence of the leukocytosis-promoting factor in inflammatory

the animals already had a leukocytosis prior to the injection of the material (Guinea pigs Nos 17 58 and 2 32, Table IV).

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SECTION MEETINGS

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IOWA	State University of Iowa February 26, 1946
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Sulfonamide Susceptibility of Stock Strains of Dysentery Bacilli and of
Strains from Recent Epidemics

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The extensive use of sulfonamides in the treatment of bacillary dysentery suggests the need for a suitable method of estimating *in vitro* the susceptibility of shigellae to the bacteriostatic action of these drugs. Most of the reports on the action of sulfonamides on *Shigella* have been concerned with comparisons of the bacteriostatic or bactericidal power of different sulfonamides when tested against a relatively small number of strains. Comparisons of the sensitivity of strains of dysentery bacilli used by different investigators are difficult to draw because there has been an almost complete lack of uniformity in the technical procedures employed. It

has been pointed out by MacLeod and Mirick¹ that a desirable test of sulfonamide susceptibility is one "which is relatively simple to perform, clear and easy to read and [one] with which readily reproducible results can be obtained." The type of culture medium, the number of bacteria in the inoculum, and the time of incubation have been shown by Cooper and Keller² to affect the results of tests of sulfonamide susceptibility.

¹ MacLeod, C. M., and Mirick, G. S., *J. Bact.*, 1942, **44**, 277.

² Cooper, M. L., and Keller, Helen M., *Proc. Soc. Exp. Biol. and Med.*, 1942, **50**, 148.

TABLE I
Results of Sulfadiazine Titrations

Series	Type of Shigella	No of strains	Lowest concentration of sodium sulfadiazine which completely inhibited growth $\times 10,000$
A	<i>Sh. paradysenteriae</i> Flexner V (I)	8	M/8
	" " " " " "	3	M/16
	" " " " " "	6	M/32*
	" " " " W (II)	1	M/4
	" " " " " "	5	M/8
	" " " " " "	3	M/16
	" " " " " "	6	M/32*
	" " " " Z (III)	1	M/2
	" " " " " "	4	M/4
	" " " " X	1	M/8
	" " " " Y	1	M/32*
	" " " Boyd 103	1	M/4
	" " " " " "	8	M/8
	" " " " " "	3	M/16
	" " " " " "	1	M/32*
	" " " " P119	1	M/8
	" " " " " "	2	M/16
	" " " " " "	3	M/32*
	" " " " 88	7	M/8
	" " " " " "	4	M/16
	" " " " " "	1	M/32*
	" " " " 170	1	M/16
	" " " " P288	2	M/8
	" " " " D1	1	M/8
	" " " " " "	1	M/16
	" " " " P143	1	M/2
	" " " " " "	3	M/4
	" " " " " "	1	M/8
	" " " " P274	3	M/8
	" " " " D19	2	M/8
	" " " " " "	1	M/32*
	" <i>sonnei</i>	3	M/2
	" " " "	4	M/4
	" " " "	7	M/8
	" <i>dysenteriae</i> (Singr)	3	M/4
	" " " "	4	M/8
	" " " "	1	M/16
	" <i>ambigua</i>	7	M/8
	" " " "	2	M/16
	" sp <i>Sacchi</i> Q771	4	M/8
	" " " " Q902	2	M/8
	" " " " " "	1	M/16
	" " " " " "	2	M/32*
	" " " " Q1030	4	M/8
	" " " " " "	1	M/16
	" " " " " "	3	M/32*
	" " " " Q1167	3	M/4
	" " " " " "	1	M/8
	" " " " Q454	1	M/4
	" <i>altalascens</i>	3	M/4
	" " " "	3	M/8
	" " " "	1	M/16
	" <i>ceylonensis</i>	9	M/8
	" " " "	2	M/16
	" " " "	1	M/32*
	" <i>madampensis</i>	1	M/8
B	" <i>paradysenteriae</i> , Flexner V (I)	13	M/8
	" " " " " "	3	M/16
	" " " " Boyd 103	1	M/8
	" <i>sonnei</i>	7	M/4
	" <i>ambigua</i>	1	M/4
	" " " "	6	M/8

of dysentery bacilli. In the present study an inhibitor-free medium which permits rapid and heavy growth of most of the strains of dysentery bacilli thus far tested has been devised. The medium and its use in the detection of sulfonamide resistant strains are described.

Cooper and Keller,³ after reviewing the earlier literature on the subject, presented the results of a study of the susceptibility *in vitro* of *Sh paradysenteriae* (Flexner) and *Sh sonnei* to sodium sulfathiazole and sulfaguanidine. In another communication,⁴ these authors reported the development *in vitro* of sulfonamide resistant strains of the above types. Hardy⁵ reported that cultures of dysentery organisms isolated from human infections which had been treated with sulfonamide for 7 or more days were commonly found to be highly resistant to sulfonamide. He observed that difficulty in treating successfully *Sh sonnei* infections appeared to be related to the readiness with which this organism develops resistance.

Culture Medium The indispensability of nicotinic acid, or related compounds, for the growth of dysentery bacilli has been demonstrated by Koser, Dorfman, and Saunders⁶ and others, and the requirement for pantothenic acid by certain strains of *Sh paradysenteriae* (Flexner) has been pointed out by Weil and Black.⁷ The medium used in the present study consisted of an acid hydrolysate of casein supplemented with tryptophane, nicotinic acid, pantothenic acid, thiamin, glucose and inorganic salts. Thiamin was included in the medium because a single strain of *Sh dysenteriae* was encountered which failed to grow in medium to which this vitamin was not added. The growth of a number of other strains of *Shigella* was

found to be unaffected by the presence or absence of added thiamin.

The medium was prepared in lots of 4 liters by dissolving in several hundred cc of distilled water, 40 g acid hydrolyzed casein (Casamino Acids Difco), 12 g anhydrous KH_2PO_4 , 72 g $\text{Na}_2\text{HPO}_4 \cdot 12\text{H}_2\text{O}$, and 8 cc salt mixture. The pH was adjusted to 7.5 with 20% NaOH. After bringing the volume to one liter, the solution was boiled for 10 minutes and filtered through paper. (Sufficient salt mixture for 50 liters of medium contains 22.5 g $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 50 mg $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$, 50 mg $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$, 50 mg $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$, 20 mg $\text{MnCl}_2 \cdot 4\text{H}_2\text{O}$, 1 cc conc HCl, and distilled H_2O to bring the volume to 100 cc).

To the filtrate was added 160 mg tryptophane, 4 mg nicotinic acid, 4 mg calcium pantothenate and 4 mg thiamin. The volume was brought to 4 liters, the medium distributed in flasks in 500 cc amounts, and then autoclaved. After autoclaving, 5 cc sterile 20% glucose was aseptically added to each flask. If a precipitate formed as a result of autoclaving, the medium was allowed to stand, and the clear supernatant fluid was aseptically transferred to another flask.

Visual examination of the degree of turbidity which developed in inoculated tubes showed that the medium supported rapid and heavy growth of the vast majority of strains tested. No diminution in the growth attained in cultures was observed upon serial subcultivation in the medium. Enumeration, by means of pour plates, of the bacterial populations of 16- to 24-hour cultures of *Sh ambigua*, *Sh sonnei*, *Sh paradysenteriae* (Boyd 103), and *Sh paradysenteriae* (Flexner V) indicated that the cultures contained 240, 400, 480 and 560 million viable organisms per cc respectively.

Technic of Titrations Stock solutions of sodium sulfadiazine were prepared by dissolving 1 g of drug in 500 cc medium, and sterilizing the resulting solution by Seitz filtration. A series of 2-fold dilutions ranging from M/125 to M/1000 and from M/1250 to M/320,000 was prepared directly in the medium using aseptic technic. Five cc amounts of the dilutions were distributed

³ Cooper, M. L., and Keller, Helen M., *J. Pediatrics*, 1943, **22**, 418.

⁴ Cooper, M. L., and Keller, Helen M., *Proc. Soc. Exp. Biol. and Med.*, 1943, **52**, 92.

⁵ Hardy, A. V., *U. S. Pub. Health Rep.*, 1945, **60**, 1037.

⁶ Koser, S. A., Dorfman, A., and Saunders, F., *Proc. Soc. Exp. Biol. and Med.*, 1940, **43**, 391.

⁷ Weil, A. J., and Black, J., *Proc. Soc. Exp. Biol. and Med.*, 1944, **53**, 24.

TABLE I
Results of Sulfadiazine Titrations

Series	Type of Shigella	No of strains	Lowest concentration of sodium sulfadiazine which completely inhibited growth $\times 10,000$
A	<i>Sh paradysenteriae</i> , Flexner V (I)	8	M/8
	" " " " "	3	M/16
	" " " " "	6	M/32*
	" " " " W (II)	1	M/4
	" " " " "	5	M/8
	" " " " "	3	M/16
	" " " " "	6	M/32*
	" " " " Z (III)	1	M/2
	" " " " "	4	M/4
	" " " " X	1	M/8
	" " " " Y	1	M/32*
	" " Boyd 103	1	M/4
	" " " "	8	M/8
	" " " " "	3	M/16
	" " " " "	1	M/32*
	" " " " P119	1	M/8
	" " " " "	2	M/16
	" " " " "	3	M/32*
	" " " " SS	7	M/8
	" " " " "	4	M/16
	" " " " "	1	M/32*
	" " " " 170	1	M/16
	" " " " P288	2	M/8
	" " " " D1	1	M/8
	" " " " "	1	M/16
	" " " " P143	1	M/2
	" " " " "	3	M/4
	" " " " "	1	M/8
	" " " " P274	3	M/8
	" " " " D19	2	M/8
	" " " " "	1	M/32*
	" sonnei	3	M/2
	" " "	4	M/4
	" " "	7	M/8
	" dysenteriae (Shiga)	3	M/4
	" " "	4	M/8
	" " "	1	M/16
	" ambigua	7	M/8
	" " "	2	M/16
	" sp Sachs Q771	4	M/8
	" " " Q902	2	M/8
	" " " " "	1	M/16
	" " " " "	2	M/32*
	" " " " Q1030	4	M/8
	" " " " "	1	M/16
	" " " " "	3	M/32*
	" " " " Q1167	3	M/4
	" " " " "	1	M/8
	" " " " Q454	1	M/4
	" allallescens	3	M/4
	" " "	3	M/8
	" " "	1	M/16
	" ceylonensis	9	M/8
	" " "	2	M/16
	" " "	1	M/32*
	" madampensis	1	M/8
B	" paradysenteriae, Flexner V (I)	13	M/8
	" " " " "	3	M/16
	" " Boyd 103	1	M/8
	" sonnei	7	M/4
	" ambigua	1	M/4
	" " "	6	M/8

TABLE I (Continued)

Series	Type of <i>Shigella</i>	No of strains	Lowest concentration of sodium sulfadiazine which completely inhibited growth $\times 10,000$
C	<i>Sh. paradysenteriae</i> , Flexner Z (III)	6	†
	" "	1	M/8
	" "	2	M/16
	" <i>sonnei</i>	5	†
	" "	2	M/2
	" "	2	M/4
D	" <i>paradysenteriae</i> , Flexner (VIII)	3	†
E	" " " W (II)	3	†
F	" " " VZ (I-III)	4	M/2
IG	" " " W (II)	1	M/2
Total		219	

* Lowest concentration tested

† Highest " " " is M/125, incomplete inhibition of growth

among standard 15 mm \times 150 mm culture tubes

In testing the bacteriostatic effect of sulfonamides various authors have shown that the endpoint of the titration is affected by the number of organisms contained in the inoculum. In the present study, preliminary experiments showed that a 10-fold decrease in the number of organisms in the inoculum frequently changed the endpoint of the titration by one tube in the direction of higher dilutions of sulfonamide. This finding served to emphasize the necessity of controlling the size of the inoculum. The inoculum employed was prepared by mixing one standard loopful of an overnight culture, using the medium described, with 20 cc sterile medium. One-tenth cc of the resulting dilution was inoculated into each 5 cc of test medium. The standard loop was made of 24 gauge platinum wire bent in the form of a circle having an inside diameter of 2 mm. The inoculum used represented approximately 1/200,000 cc of culture, and contained of the order of 2500 viable organisms.

The tubes were examined for growth after incubating for 16 to 20 hours at 37°C. The highest dilution of sodium sulfadiazine which completely inhibited growth was chosen as the endpoint of the titration.

Strains of Dysentery Bacilli Information concerning the original sources of the strains is presented in connection with the results. Grateful acknowledgment is made to Captain

C V Seastone of the Army Medical Center for supplying us with the cultures of Series A, to Doctor A V Hardy of the United States Public Health Service for the cultures of Series B and C, and to Commander L A Barnes, H (S), USNR, of the National Naval Medical Center for cultures of Series D, E, and F. Doctor Hardy and Doctor Barnes kindly supplied epidemiological information concerning strains.

Results and Discussion 230 strains of *Shigella* were tested for their susceptibility to the bacteriostatic action of sodium sulfadiazine. The results of the tests are presented in Table I. It is evident that the strains fall into one of 2 categories in regard to their sensitivity to the sulfonamide: (1) Strains whose growth was inhibited by a drug concentration of M/20,000 or less, (2) strains which grew in the presence of a drug concentration as great as M/125. Of 219 strains, 202 belong to the first category and may be designated as susceptible strains, 17 belong to the second category and may be designated as resistant strains. Eleven strains failed to show optimal growth in control tubes containing no sodium sulfadiazine, and for this reason tests of the susceptibility of these strains have been omitted from the tabulation.

Series A consisted of 159 strains from the culture collection of the Army Medical School. This series contained one or more representative strains of each of the well established

species of *Shigella*. It is notable that without exception the strains of this series were of the susceptible variety since growth was inhibited by a concentration of sodium sulfadiazine of 1/20,000 or less. Although growth of some of the strains was prevented by a concentration as low as 1/320,000 while other strains required as much as 1/20,000 for inhibition of growth, there appeared to be little or no correlation between sulfonamide sensitivity and species or type of *Shigella*.

Series B consisted of 31 strains isolated from inmates of an institution in Illinois. With the exception of 2 cases, there was no clinical evidence to suggest the existence of sulfonamide resistance. The 2 exceptions were children who were reported to be intermittently positive for *Sh. sonnei* and *Sh. ambigua* respectively, for a period of several months. Table I shows that all of the strains of this series were susceptible to sodium sulfadiazine.

Series C consisted of 9 strains of *Sh. paradysenteriae* Flexner Z and 9 strains of *Sh. sonnei*, all of which were isolated from patients in various institutions in New York. Information which has been supplied to us indicates that the patients failed to respond to sulfonamide therapy. Table I shows that 6 of the *Sh. paradysenteriae* Flexner Z strains and 5 of the *Sh. sonnei* strains were sulfonamide-resistant.

Series D consisted of 3 strains designated as predominantly Flexner VIII. These strains were isolated from patients who failed to respond to sulfadiazine therapy. One of the strains appeared in epidemic form among the personnel of a naval vessel subsequent to sailing from Okinawa. The epidemic is reported to have flared up at a later date at Sagami Bay. All known methods of curbing carriers were said to have been applied without success. The other 2 strains of this series were 2 isolations from an individual who became a carrier following an epidemic on board a naval vessel in the vicinity of Kulambangara, Solomon Islands. The 2 strains were isolated nearly a year after the appearance of the epidemic, and the opinion

has been expressed⁸ that they do not represent the etiologic agent in the epidemic since 4 other strains from persons on the same vessel were *Sh. paradysenteriae* Flexner W. Table I shows that all 3 strains were sulfonamide-resistant.

Series E consisted of 3 strains of *Sh. paradysenteriae* Flexner W isolated during an outbreak of dysentery which occurred on a naval vessel in the Pacific. The epidemic involved more than 300 men, and there were 2 fatalities. Table I shows that these strains were sulfonamide-resistant.

Series F consisted of 5 strains, 4 of which were isolated during an outbreak on a vessel which had been anchored at a harbor in the Marshall Islands. The fifth strain (*Sh. paradysenteriae* Flexner W) was isolated during the course of an outbreak on a vessel which had sailed from Iwo Jima. Table I shows that all 5 of these strains were sulfonamide-susceptible. Information is lacking concerning the response of the patients to sulfonamide therapy.

Although the present study affords little indication of the precise frequency with which sulfonamide-resistant infections may be expected to be encountered, it nevertheless serves to demonstrate by cultural means the existence of such infections. The occurrence of cases of dysentery failing to respond to sulfonamide and yielding organisms which exhibit *in vitro* a high degree of drug-resistance suggests the desirability of performing sulfonamide-susceptibility tests on a greater scale than has been done hitherto. It would appear that in the future sulfonamide-resistant organisms may constitute a serious threat to the successful treatment of dysentery.

Summary. A method of testing the sensitivity of dysentery bacilli to the bacteriostatic action of sodium sulfadiazine is described. The method involves the use of a medium which is essentially free of sulfonamide inhibitors. Of 219 strains tested, 202 were found to be susceptible to the drug while 17 strains were found to be resistant. Among the susceptible strains were representatives of each of the well established varieties of

⁸ Barnes, L. A., personal communication.

Shigella The resistant strains included representatives of *Sh paradysenteriae* (Flexner types W, Z and VZ) and *Sh sonnei*. Many of the resistant strains were isolated from

patients who failed to respond to treatment with sulfonamides. The results serve to emphasize a potential difficulty in the chemotherapy of dysentery.

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Comparative Study of Hypertensinase and Proteinase Activity of Blood Plasma

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Hypertension is inactivated by proteolytic ferments (Muñoz and co-workers)¹. This has been demonstrated by the action of crystalline enzymes. Inactivation of hypertensin with pepsin was accomplished by Alonso, Crovatto and Crovatto,² with trypsin and chymotrypsin by Crovatto, Crovatto, Illanes and Salvestrini,³ and with carboxypeptidase by Plentl and Page.⁴ Purified aminopeptidase, obtained from yeast, inactivates hypertensin rapidly. Hypertensinase extracts of kidney and other organs, when examined for their proteinase activity, exhibit characteristics typical of aminopeptidase at a pH near the neutral point (Crovatto and Crovatto).⁵ Aminopeptidase, obtained from yeast, and hypertensinase extracts of fresh tissues, red blood cells, blood plasma, etc., show a similar behavior toward oxytocin and vasopressin. Both pituitary hormones are inactivated by the various preparations in the presence of an excess of SH groups added in the form of cysteine or glutathione. The rapidity of their action is due to the hypertensinase ac-

tivity peculiar to them (Crovatto and Ebensperger).⁶

It is well known that plasma possesses hypertensinase activity which, though slight, is easy to demonstrate. There are certain indications for the assumption that plasma hypertensinase is of major importance. Even when hypertensin is present in the plasma it cannot enter into the red blood cells. Recent experiments have shown that complete ligation of the arteries of certain parenchymatous organs such as kidneys and liver, has little effect on the pressor response produced by continuous or interrupted injections of hypertensin or renin (Crovatto and Neira).⁷ From these considerations, it seemed of interest to investigate the proteolytic properties of plasma as well as its hypertensinase activity under physiologically normal conditions of pH and temperature.

According to Bergmann and his co-workers⁸ studies of proteinase specificity can be accomplished quantitatively by using as substrates only a few synthetic polypeptides of known structure, which are hydrolyzed to peptides. Accordingly, we have used the following polypeptides: l-leucyl-glycine, dl-leucylidiglycine, dl-methylleucylidiglycine, carbobenzoxy-glycyl-phenylalanine, alpha-benzoyl-

¹ Muñoz, J. M., Bruun Menendez, E., Fasciolo, J. C., and LeLoir, L. F., *Amer J Med Sci*, 1940, **200**, 608.

² Alonso, O., Crovatto, R., and Crovatto, H., *Proc Soc Exp Biol and Med*, 1943, **52**, 61.

³ Crovatto, H., Crovatto, R., Illanes, G., and Salvestrini, H., *Bol Soc Biol*, Santiago, Chile, 1942, **1**, 4.

⁴ Plentl, A., and Page, I., *J Exp Med*, 1944, **79**, 205.

⁵ Crovatto, R., and Crovatto, H., *Science*, 1942, **96**, 519.

⁶ Crovatto, H., and Ebensperger, A., *Bol Soc Biol*, Santiago, Chile, 1944, **2**, 205.

⁷ Crovatto, H., and Neira, E., *Bol Soc Biol*, Santiago, Chile, 1944, in press.

⁸ Fruton, T. S., Irving, G. W., and Bergmann, M., *J Biol Chem*, 1941, **138**, 249.

TABLE I
Proteolytic Action of Plasma from Various Species

Plasma	Amount used cc	Time of incubation hr	Substrate (0.05 M)	Hydro- lysis %	pH
Human (A)	2	24	l-leucylglycine	30	7.6
(B)	2	24	"	25	"
(C)	2	22	"	23	"
(D)	2	23	"	24	7.8
(E)	2	24	"	30	"
(F)	2	24	"	47	7.6
Bovine (1126)	0.2	24	"	4	7.5
(1126)	2	22	"	63	7.8
(1159A)	2	24	"	28	7.6
(1159B)	2	25	"	20	7.8
(1154)	2	24	"	34	7.6
(1141)	2	24	"	3	"
Equine (1160A)	2	24	"	46	"
Feline (1869)	2	24	"	85	"
(1869B)	2	24	"	95	"
(1931)	2	24	"	58	"
(1931)	2	24	"	50	"
(1931B)	2	24	"	34	"
Human (A)	2	27	carbobenzoxy-glutamyl phenylalanine	—	"
(B)	2	27	"	—	"
Bovine (1126)	2	23	"	40	7.8
(1154)	2	24	"	—	7.6
Feline (1869)	2	24	"	—	"
(1931)	2	24	"	3	"

Benzoyl-L-arginineamide and carbobenzoxy-L-glutamyl-L-tyrosine were not hydrolyzed by human, bovine and feline plasmas

l-arginineamide and carbobenzoxy-glutamyl-L-tyrosine, these being substrates for determining the presence of aminopeptidase, carboxypeptidase, trypsinase and pepsinase. An analysis of chymotrypsinase activity had to be omitted for lack of an adequate substrate.

Methods Arterial blood of cats, cows, and horses, and human venous blood were used. The blood was collected directly in a centrifuge tube containing 3.8% sodium citrate solution in the proportion of 1 to 10. Precautions were taken to avoid hemolysis. The centrifuged plasma was mixed in amounts varying between 0.2 and 2 cc with the various peptides, the hydrolysis of which was measured by the method of Grassman and Heyde,⁹ the titration being done 20 to 40 hours after addition of reagents. To prevent troublesome precipitation of albumin from the 2 cc of plasma, the albumin was removed by centrifugation after adding the optimum

amount of alcohol required for the titration. The clear liquid was then titrated exactly according to the methods described. In all cases the concentration of substrate was 0.05 M dissolved in a 1 M/15 to M/30 buffered phosphate solution at a pH ranging from 7.3 to 7.8. The free carboxyl groups were determined by adding 0.01 N alcoholic KOH solution. The values obtained in the blank were subtracted. The percentage of hydrolysis was calculated by parallel measurements on mixtures of peptides and amino acids in equimolecular proportions and by using crystallized trypsin and carboxypeptidase with their corresponding substrates. In all experiments sterile material was employed and strictest precautions of asepsis were observed. Frequently the plasma was sterilized by passage through a Seitz filter.

Results As will be seen in Table I, the estimation of hydrolysis was difficult with the use of minute amounts of plasma, but with a ratio of 2 cc of plasma to 1.36 cc of substrate, an appreciable hydrolysis of l-leucylglycine was obtained within the in-

⁹ Grassmann, W., and von Heyde, W. Z., *Physiol. Chem.* 1929, **183**, 32.

TABLE II
Proteolytic Activity of Various Fractions of Plasma Obtained by Precipitation with Ammonium Sulphate

Plasma (fraction)	Amt used cc	Time of incubation hr	Substrate (0.05 M)	Hydrolysis %	pH
1083 AG	0.2	22	l-leucylglycine	28	7.3
" "	0.4	26	"	63	7.6
" AA	0.4	26	"	20	7.4
1154	2.0	24	"	36	7.6
" A	2.0	24	"	38	"
" B	2.0	24	"	49	"
1083 AG	0.2	22	dl-leucyl diglycine	24	7.4
" "	0.2	44	"	56	"
" "	0.2	48	dl-methyl-leucyl diglycine	—	"
" "	0.4	24	carbobenzoyl-glycyl-phenylalanine	25	"
" "			carbobenzoyl-glycyl-		
" AA	0.4	48	phenylalanine	3	"
" "	0.4	24	benzoyl-L-arginineamide	6	"

These results correspond with the letters H, I, and J on Fig. 1. 3 mg of cysteine were added. Compare with Fig. 2. Fraction 1083AG was obtained by adding a saturated solution of ammonium sulphate drop by drop to half saturation. Fraction 1083AA represented an albumin fraction obtained by full saturation with ammonium sulphate after removing the previous fraction. The ammonium sulphate was removed by dialysis.

icated time periods. Benzoyl-arginineamide and carbobenzoyl-glutamyltyrosine were not hydrolyzed by plasma. Carbobenzoyl-glycyl-L-phenylalanine was partially hydrolyzed by bovine plasma in one case.

Plasmas kept near 0°C for a period of sev-

eral weeks retained their ability to split off leucylglycine.

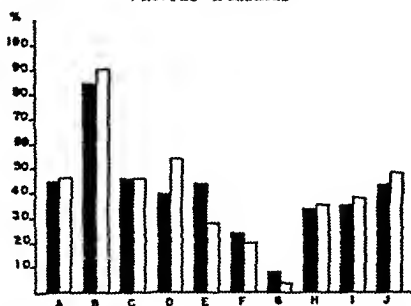
The hypertensinase activity of the plasmas bore a close relation to their hydrolyzing effect on l-leucylglycine. In Fig. 1 the % destruction of 2 units of hypertensin by 1 cc of plasma after 2 hours of incubation at 37°, at a pH of 7.4 is shown. The action of aminopeptidase activity was measured in 2 cc of plasma for 1.36 cc of 0.05 M l-leucylglycine at a pH of 7.3 and 7.8 after 24 hours of incubation at 37°.

The treatment of plasma with various concentrations of ammonium sulphate and its subsequent dialysis yields fractions with varying degrees of hydrolytic activity for l-leucylglycine (Table II). The hypertensinase activity of these various fractions closely parallels the hydrolyzing effect of the plasma on l-leucylglycine (Fig. 2). Heating the plasma to 58° and acidifying it to pH 3.2 decreases equally its power to inactivate hypertensin and its aminopeptidase action (Fig. 3).

Discussion. Plentl and Page,¹⁰ by studying the kinetics of the destruction of hypertensin by hypertensinase, found that it fulfilled the characteristics of a first-order reaction. Studies

¹⁰ Plentl, A., and Page, I., *J. Exp. Med.*, 1943, 78, 367.

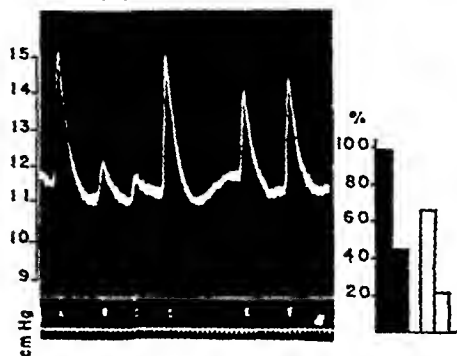
Fig. 1
Hypertensinase and Aminopeptidase Activity of Various Plasmas



Black columns: Hypertensinase activity expressed in percentage destruction of the hypertensin; white columns: aminopeptidase activity. A, human plasma F, B, feline plasma 1869B (passed through a Seitz filter), C, equine plasma 1160A, D, feline plasma 1931, E, bovine plasma 1159A, F, bovine plasma 1159B, G, bovine plasma 1151, H, bovine plasma 1154, I, bovine plasma 1154 (fraction A), J, bovine plasma 1154 (fraction B). Fractions A and B were obtained by precipitation with half and complete saturations of ammonium sulphate respectively and subsequent dialysis. Each column represents the average of 3 separate determinations.

Fig 2

Aminopeptidase and Hypertensinase Activity of Fractions 1083AG and 1083AA Blood Pressure of a Cat Anesthetized with Dial



The following mixtures were injected intravenously after 5 hours of incubation at 37° at pH 7.4. At A, 2 units of hypertensin; at B, 2 units of hypertensin plus 0.05 cc of fraction 1083AG; at C, 2 units of hypertensin plus 0.2 cc of fraction 1083AG; at D, 2 units of hypertensin plus 0.05 cc of fraction 1083AA; at E, 2 units of hypertensin plus 0.2 cc of fraction 1083AA; at F, 2 units of hypertensin.

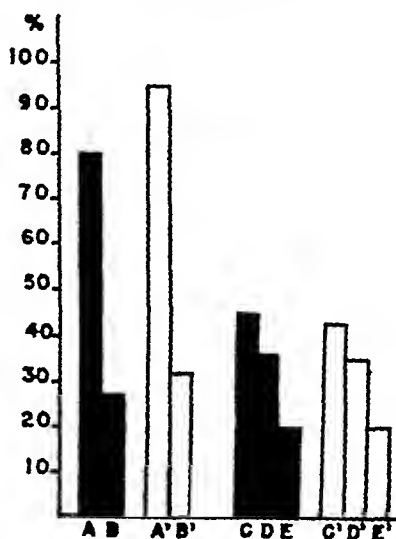
Figure on the right: Black columns: Percentage destruction of hypertensin by fractions 1083AG and 1083AA respectively after 5 hours of incubation. White columns: Percentage break down of 1-leucylglycine after 24 hours of incubation (136 cc solution 0.05 mM, pH 7.8) in the presence of 0.1 cc of fractions 1083AG and 1083AA respectively.

on the hydrolysis of 1-leucylglycine and on the inactivation of hypertensin by aminopeptidase suggests a similar type of reaction for both (Valenzuela).¹¹ The magnitudes of both reactions show a close parallelism. These observations and the data obtained on the behavior of aminopeptidase and hypertensinase extracts toward oxytocin and vasopressin strongly support the hypothesis that the hypertensinase activity of plasma is identical with that of aminopeptidase. A systematic investigation is being made of tissue extracts and slices.

Summary This study supports the hypothesis that the hypertensinase activity

Fig 3

Hypertensinase and Aminopeptidase Activity of Human and Feline Plasmas, Before and After Heat and Acidification



Black columns: Hypertensinase activity. White columns: Aminopeptidase activity.

A, percentage destruction of 2 units of hypertensin by 1 cc of feline plasma after 2 hrs incubation. B, same as A, except for previous heating of plasma at 58°, 90 min. A', percentage break down of 1-leucylglycine (136 cc 0.05 mM) by 2 cc of feline plasma after 24 hrs incubation. B', same as A', except plasma subjected to same procedure as in B. C, percentage destruction of 2 units hypertensin by 1 cc human plasma after 2 hrs incubation. D, same as C, except for previous heating of plasma to 58°, 60 min. E, same as C, except acidifying the plasma to a pH of 3.2 for 60 min and subsequent neutralization. C', percentage break down of 1-leucylglycine (136 cc 0.05 mM) by 2 cc of human plasma after 24 hrs incubation. D', same as C, except subjecting plasma to procedure as in D. E, same as C, except subjecting the plasma to the same procedure as in E.

of plasma at a pH of 7.3 to 7.8 and at a temperature of 37° is closely related to its exopeptidase activity, its nature being that of aminopeptidase.

Acknowledgment is made to Prof M. Bergmann for his generous advice. Some of the peptides used in these studies are being sent to Drs T. S. Fruton and A. Plentl.

¹¹ Valenzuela, B. *Tesis*, Universidad, Chile, 1942.

TABLE II
Proteolytic Activity of Various Fractions of Plasma Obtained by Precipitation with Ammonium Sulphate

Plasma (fraction)	Amt used cc	Time of incubation hr	Substrate (0.05 M)	Hydrolysis %	pH
1083 AG	0.2	22	l-leucylglycine	28	7.3
" "	0.4	26	"	63	7.6
" AA	0.4	26	"	20	7.4
1154	2.0	24	"	36	7.6
" A	2.0	24	"	38	"
" B	2.0	24	"	49	"
1083 AG	0.2	22	dl-leucyl diglycine	24	7.4
" "	0.2	44	"	56	"
" "	0.2	48	dl-methyl-leucyl diglycine	—	"
" "	0.4	24	carbobenzoyl-glycyl-phenylalanine	25	"
" "	"	"	carbobenzoyl-glycyl-l-phenylalanine	3	"
" AA	0.4	48	benzoyl-l-arginineamide	6	"
" "	0.4	24	"	6	"

These results correspond with the letters H, I, and J on Fig. 1. 3 mg of cysteine were added. Compare with Fig. 2. Fraction 1083AG was obtained by adding a saturated solution of ammonium sulphate drop by drop to half saturation. Fraction 1083AA represented an albumin fraction obtained by full saturation with ammonium sulphate after removing the previous fraction. The ammonium sulphate was removed by dialysis.

icated time periods. Benzoyl-arginineamide and carbobenzoyl-glutamyltyrosine were not hydrolyzed by plasma. Carbobenzoyl-glycyl-l-phenylalanine was partially hydrolyzed by bovine plasma in one case.

Plasmas kept near 0°C for a period of sev-

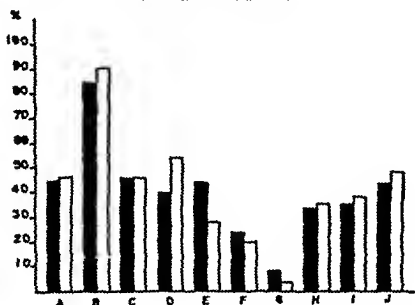
eral weeks retained their ability to split off leucylglycine.

The hypertensinase activity of the plasmas bore a close relation to their hydrolyzing effect on l-leucylglycine. In Fig. 1 the % destruction of 2 units of hypertensin by 1 cc of plasma after 2 hours of incubation at 37°, at a pH of 7.4 is shown. The action of aminopeptidase activity was measured in 2 cc of plasma for 1.36 cc of 0.05 M l-leucylglycine at a pH of 7.3 and 7.8 after 24 hours of incubation at 37°.

The treatment of plasma with various concentrations of ammonium sulphate and its subsequent dialysis yields fractions with varying degrees of hydrolytic activity for l-leucylglycine (Table II). The hypertensinase activity of these various fractions closely parallels the hydrolyzing effect of the plasma on l-leucylglycine (Fig. 2). Heating the plasma to 58° and acidifying it to pH 3.2 decreases equally its power to inactivate hypertensin and its aminopeptidase action (Fig. 3).

Discussion. Plentl and Page,¹⁰ by studying the kinetics of the destruction of hypertensin by hypertensinase, found that it fulfilled the characteristics of a first-order reaction. Studies

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¹⁰ Plentl, A., and Page, I., *J. Exp. Med.*, 1943, 78, 367.

TABLE I
Sources of Cultures

Source	Rettgeri	Eberthellae
Human diarrhea	23	3
" carriers	9	3
" urine	1	1
Monkey	3	3
Rabbit	2	1
Guinea pig	1	0
Hog	1	0
Unknown	4	3
Total	44	13

Vaccaro *et al*¹⁶ isolated 5 species of *Eberthella*, including *belfastensis*, *enterica*, and *oedematensis*. The last named species was also observed recently by Fuentes *et al*¹⁷ and by Neter¹⁸

Eberthella strains have been isolated from sources other than the human intestine. Thus Peterson¹⁹ described 3 species found in well water. Duthie and Mitchell²⁰ found eberthellae in pseudotuberculosis of rodents. Emmel²¹ described various eberthellae from parasite-infested fowl.

It is the purpose of the present paper to present a more complete description of Rettger's bacillus than has hitherto been published, and to discuss the relationship between this species and the rarer strains having generic similarities to it.

Observations. The material studied in this work was a collection of 57 strains of gram negative bacilli which were anaerogenic or microaerogenic, motile, and lactose negative. The sources of the cultures are indicated in Table I. The chief feature of this table is its evidence that these bacilli occur in animals as well as in man. The cultures from monkeys were described previously by Pres-

ton and Clark²² and kindly submitted by Preston. The cultures of unknown origin are stock cultures whose source could not be traced.

No less than 44 of these cultures were identified as Rettger's bacillus. The characteristics of this species are fully set forth in Table II. All 44 strains conform quite closely to the reactions set forth although occasionally strains may exhibit one or 2 negative reactions in tests that are regularly positive. Rustigian and Stuart⁹ showed that some strains ferment salicin, others do not. In the present series 23 were salicin positive and 21 negative. This was also true for rhamnose which was fermented by 29 strains, not fermented by 15. Occasionally a strain was encountered which failed to conform to the description in one or 2 respects. For example, 3 strains were urea negative, one citrate negative, one glycerol negative, and tests confirmed the absence of motility in a strain received from Stuart although all our own isolations were motile.

Colonies of Rettger's bacillus are rather distinctive on certain plating media. The greenish-brown, mucoid colonies on bismuth sulfite agar are very characteristic. Less so but still unusual, are the colonies on S-S agar, where brown centers develop which are not at all similar to the intense blackening of *Salmonella* and *Protus* colonies. No swarming of colonies has been observed on any plating medium, although Rustigian and Stuart^{8,9} have reported its occurrence under special conditions.

The motility of this species, originally recorded by Hadley as negative, was demonstrated by Rustigian and Stuart⁸. The difficulty which they appear to have had in demonstrating motility has never occurred in tests using freshly prepared Bacto Motility Test Medium, incubated at 37°C. Bergey describes *Shigella rettgeri* as xylose positive, only one strain of the 44 fermented this pentose. Single strains also fermented cellobiose or maltose or trehalose. Sucrose is fermented after several days when its con-

¹⁶ Vaccaro, H., Ibarra Loring, E., Perez, M. M., and Mancilla, *Rev. Med. Chile*, 1943, **71**, 495.

¹⁷ Fuentes, C., Angulo, J. J., and Madrido, O., *Rev. Med. Trop. Parasit*, 1943, **9**, 22.

¹⁸ Neter, E., *Arch. Path.*, 1939, **28**, 122.

¹⁹ Peterson, O. H., *Thesis, Univ. of Missouri*, 1942.

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MACDONALD FULTON AND S F CURTIS

From the Department of Pediatrics, University of Texas School of Medicine, Galveston

Few reports of the occurrence of Rettger's bacillus can be found in the literature prior to its isolation by Cope and Kilander¹. Originally the organism was isolated by Rettger during his studies of diseases of fowl. It was described, named and classified by Hadley, Elkins and Caldwell,² by whom it was considered to be a member of the genus *Shigella*. St John Brooks and Rhodes³ studied one strain obtained from Hadley's collection, but aside from noting that it produced indol, they made no mention of its characteristics. The species is assigned to the genus *Shigella* in Bergey's Manual, Vth Edition, where it was originally placed by Bergey and later by Weldin⁴. Neter⁵ in reviewing the genus *Shigella* called attention to the scanty and contradictory knowledge of this species. Very shortly, Cope and Kilander in the aforementioned paper rediscovered the species in human gastroenteritis. At about the same time, Stuart and co-workers⁶ encountered strains called "type 33111" in their study of the paracolon group.

Rustigian and Stuart⁷ in the following year suggested the name "*Proteus entericus*" for these strains. Subsequently these workers⁸ secured presumably authentic type strains and

demonstrated that the true species name for their "type 33111" and "*Proteus entericus*" was "*rettgeri*." They assigned the species to the genus *Proteus*. Their conclusions, based on the study of 78 strains are embodied in a recent paper by Rustigian and Stuart⁹.

Fulton and Curtis¹⁰ previously reported studies of this species, and Felsenfeld and Young¹¹ have isolated strains in the Chicago area. There do not appear to be other published reports of the occurrence of this organism, although its isolation in New England, Michigan, Illinois and Texas suggests that it is of widespread distribution.

Organisms resembling Rettger's bacillus but not identical with it have been isolated from time to time by most students of enteric bacteriology. Such cultures have the cardinal characteristics of the genus *Eberthella*. Fourteen species are recognized by Bergey, most of the descriptions being incomplete. The communications of de Assis¹² and Herring¹³ describe certain species of *Eberthella* but there is little published information concerning the characteristics of this group. A detailed search would undoubtedly bring to light many hidden references to isolations of *Eberthella* species, such as have been made by Costa Mandry¹⁴ and Hill *et al*¹⁵. In a study of 480 fecal specimens from adults,

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³ St John Brooks, R, and Rhodes, M, *J Path and Bact*, 1923, 26, 434

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TABLE III

Name of test	Strains				
	I	II	III	IV	V
adonitol	0	—	—	—	—
arabinose	—	—	+	+	—
cellobiose	—	—	—	—	+
dulcitol	—	—	+	—	—
galactose	+	—	+	+	+
1% glycerol	+	—	—	0	+
inositol	+	+	—	—	+
5% lactose	0	—	+	0	—
0.5% lactose	—	—	—	—	—
maltose	+	+	+	+	—
mannitol	+	—	+	+	—
mannose	+	—	+	+	+
rhamnose	—	—	+	+	—
saline	+	—	+	—	—
sorbitol	+	—	+	—	—
5% sucrose	0	—	—	0	+
0.5% sucrose	+	—	—	—	+
trehalose	—	+	+	+	+
xylitol	—	—	+	—	—
indol	—	+	+	+	+
mr	—	+	—	+	+
vp	+	—	—	—	—
citrate	+	—	—	—	+
gelatin	+	—	—	+	—

+ Positive reaction

— Negative reaction

0 Undetermined

The same 4 strains were used to adsorb the O serum. Only strain 1544 agglutinated, and antibody adsorption was complete in one treatment. Strains 517 and 519 were not agglutinated by the serum and did not adsorb any of the strain 1544 O agglutinins from it. They apparently do not belong to the same O antigenic group as strain 1544, although bacteriologically they are identical with it. Strain 571 slowly but continuously lowered the titer of strain 1544 O agglutinins although it was not itself agglutinated by the serum. It may be that this reaction was also seen by Rustigian and Stuart,⁸ they failed to mention whether they observed it with O or H antibodies or with both. This kind of adsorption reaction is by no means unique in this species. There is no generally accepted interpretation of it. It may indicate failure of the second stage of the antigen-antibody reaction, or an antigenic relationship of the partial antigen type between the strains. Certain considerations suggest that it could also result from gradual saturation of the antibody by an antigen fraction liberated from the cells by the heat employed

in preparing the O antigen suspension.

It is evident that the serological analysis of Rettger's bacillus has not been completed and that it poses some interesting problems. It is useful to know that the use of the single serum permits ready recognition of most strains, as the agglutination test is the most rapid means of identifying the type.

Strains of the genus Eberthella. Thirteen strains of gram negative bacilli were studied which were motile, anaerogenic, and moderately varied in fermentative powers. Five strains may be Rettger's bacillus but departed rather widely from the typical description and were not agglutinated by "Detroit 1544" H or O serum. Three others form a group which fermented adonitol, glucose, glycerol, levulose, mannose, and sucrose, produced indol, grew on citrate agar and alkalinized milk. One of these cultures was isolated from human diarrhea, one was a stock culture called *Alcaligenes alcalofoetidus* and one was isolated by Preston from a monkey. Although these cultures resembled Rettger's bacillus in their adonitol and indol reactions, their fermentative activity was quite restricted.

TABLE II
Characteristics of Rettger's Bacillus

adonitol	+	levulose	+	xyllose	—
arabinose	—	maltose	—	milk	alk
cellobiose	—	mannitol	+	urea	+
dextrin	—	mannose	+	indol	+
dulcitol	—	methygluc	—	mr	+
galactose	+	raffinose	—	vp	—
glucose	+	ibramnose	+ or —	citrate	+
glucose gas	—	sahem	+ or —	gelatin	—
1% glycerol	+	starch	—	motility	+
inositol	+	sorbitol	—	sulfide	—
inulin	—	5% suerose	+	BiSO ₄ greenish brown	—
5% lactose	—	0.5% suerose	—	DC, SS and Mac plates	+
0.5% lactose	—	trehalose	—	spreading on agar	—

+ Acid

— No acid

+ or — Some strains produce acid, some do not

alk Alkaline milk

centration is 5% At 1/10th this concentration acid is rarely formed A similar effect of changing the concentration of sucrose on the formation of acid has been observed with *Proteus ammoniae* and *Shigella sonnei*

The fermentation of adonitol is the most striking single characteristic of this species In a study of over 1000 strains of Enterobacteriaceae, fermentation of this polyatomic alcohol has been observed only with cultures of Rettger's bacillus, with certain paracolon strains and occasional strains of uncertain identity Of all the known species of enteric bacteria, this is the only one characterized by the tetrad of adonitol fermentation, urea hydrolysis, indol formation and growth on Simmons citrate agar Among these 4 cardinal characteristics, adonitol fermentation is the most restricted in distribution among the other gram-negative bacilli, therefore most nearly a distinctive characteristic of Rettger's bacillus

Serologically, Rettger's bacillus is distinct from the other Enterobacteriaceae but heterogeneous itself None of the strains agglutinated in any serum of the Salmonella Typing Center series, nor in any *Shigella* serum (Lederle), nor in *Proteus*, *Morgan* s bacillus and *columbensis* bacillus serums

Serums were prepared against both formalinized and boiled vaccines of strain 1544 isolated in Detroit Difficulty was experienced in securing O serum of sufficient titer, the vaccine being quite toxic for rabbits on intravenous injection The titer of the H

serum was 3200, of the O serum 2500 There was little O antibody in the H serum, but the O serum had a higher titer with raw than with boiled antigen This may indicate a lability of the O antigen which contrasts with the heat stability of this type of antigen in the Salmonella group

The majority of the cultures, 21 in number, agglutinated in both H and O serums One agglutinated in O serum but not in H, while 5 agglutinated in H but not in O Ten did not agglutinate in either serum Six cultures were too rough to use in agglutination tests Heating one-half hour at 80°C did not improve the agglutination, and in many instances decreased it Certain interesting relationships developed during antibody-adsorption experiments Antigen was prepared from thick tryptose agar plates flooded with one ml of tryptose phosphate broth culture and incubated overnight, suspended in saline and formalinized or boiled The H serum was diluted 1:50, the O serum 1:5, and 1 ml absorbed twice with the growth from single plates

Strains 1544 and 571 were agglutinated in the H serum, strains 517 and 519 were not Complete adsorption occurred with strain 1544, no change with 517 and 519 Removal of agglutinins for 571 without decrease of titer with 1544 resulted when 571 was used as adsorbing antigen Thus there appear to be strains of Rettger's bacillus having totally unlike H antigens, as well as strains having antigenic fractions in common

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Serological Reactions of Levans Formed from Sucrose and Raffinose by Certain Bacilli*

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Levans, which are water soluble polymers of fructose anhydride synthesized from sucrose and raffinose by some members of the genus *Bacillus* and also by some other bacteria, constitute a chemically well defined class of bacterial polysaccharides¹. The serological properties of the levans formed by one spore-forming rod (called bacillus N9) and of several strains of *S. salivarius* have been reported^{2,3}. The investigation of levans from a larger number of bacteria was required in order to get a more adequate concept of the serological properties of this class of bacterial polysaccharide and thus obtain a better basis for the application of serological methods to problems connected with the general subject of levan formation. Since the most widely distributed levan-forming agents are bacilli, the first step in the accumulation of the required information seemed to be to determine whether or not the levans formed by a number of species or varieties of bacilli had serological properties similar to those of the levans of the previously studied bacillus N9 and *S. salivarius*.

Materials and Methods The levan-forming bacteria were *B. subtilis* ("Marburg" strain, American Type Culture No 6051), *B. panis* (American Type Culture No 8472), 5 strains of *Bacillus* (called A to E, not identified as to species) which had been isolated from feces, air and commercial

sugars, and 3 unidentified Gram-positive non-sporulating rod-shaped bacteria[†] isolated from plants. These 10 bacteria showed features which are commonly associated with levan formation namely, the supernatant fluids from cultures grown with sucrose or raffinose had a more opalescent or mucoid appearance and a greater content of material precipitable with 2.5 volumes of alcohol than did the fluids of corresponding cultures grown with glucose or other sugars, also the sucrose and raffinose fluids contained considerable amounts of free reducing sugars. The bacteria used as controls comprised the previously studied bacillus N9 and *S. salivarius* S20B from both of which chemically identified levans had been obtained^{2,4}. 7 other strains of *Bacillus* (including *B. anthracis*) none of which showed features indicative of levan formation, and *Leuconostoc mesenteroides* B, which was known to produce dextran but no levan,^{3,5} the latter was included in order to control the adequacy of the experimental method to distinguish levans from dextrans.

The bacteria were inoculated into 3 sets

[†] Since most, if not all of the non sporulating rod shaped bacteria reported in the literature as levan formers have been gram negative varieties, the following description is given of the properties possessed in common by these gram positive strains. All were aerobic, non motile, catalase positive, grew readily on ordinary peptone mediums, liquefied gelatin gave acid from pentoses, grew better at 45°C than at 10°C, and showed no evidence of spore formation in many repeated tests on a wide variety of mediums. The 3 strains, however, differed from each other in fermentation of some carbohydrates.

* Aid was received by a grant from the Sugar Research Foundation, Inc.

¹ Smith, R. Greig, *Proc. Linn. Soc., New South Wales*, 1901, **26**, 589; Steel, T., *Proc. Linn. Soc., New South Wales*, 1901, **26**, 626. Many later papers are cited in reference 2.

² Hehre, E. J., Genghof, D. S., and Neill, J. M., *J. Immunol.*, 1945, **51**, 5.

³ Hehre, E. J., and Neill, J. M., *J. Exp. Med.*, 1946, **83**, 147.

⁴ Niven, C. F., Smiley, K. L., and Sherman, J. M., *J. Biol. Chem.*, 1941, **140**, 105.

⁵ Sugg, J. Y., and Hehre, E. J., *J. Immunol.*, 1942, **43**, 119.

and they were also serologically distinct from that species. The differential reactions of the remaining 5 cultures are listed in Table III. None of these 13 cultures was antigenically related to any *Salmonella*, *Shigella* or *Proteus* strain studied.

This group of 13 strains is fairly representative of the kinds of organisms included by Bergey in the genus *Eberthella*. Unless great weight is assigned to its ability to hydrolyze urea, Rettger's bacillus would appear to fall within this genus. Rustigian and Stuart⁹ have emphasized urea hydrolysis, and as further significant characteristics, mention the occasional production of a small amount of gas together with their observation that swarming occurs under special conditions. There is no clear reason for attempting to include in the genus *Proteus* all bacteria which hydrolyze urea, and the supporting characteristics mentioned are apparently not easy to confirm experimentally. It is desirable to take into consideration all available evidence as to the characteristics and properties of organisms whose systematic relationships are being worked out, even though for purposes of practical rapid recognition a few of the characteristics may be assigned relatively greater weight than the others.

If this view is taken, it is possible to argue that Rettger's bacillus and the other organisms here described belong to the genus *Eberthella*. They are motile, anaerogenic, parasitic, and antigenically distinct from the other Enterobacteriaceae. They have been

observed the world over by many workers, although usually they are relatively infrequent. The experience of Cope and Kilander¹ shows that one type at least has occurred with epidemic frequency. The same may happen with any of the other types.

The status of the genus *Eberthella* is at present obscure. It was created originally as a subgenus by Buchanan²³ and elevated to generic rank by Bergey in the first edition of his Manual. At that time Rettger's bacillus was classified as *Eberthella rettgeri*. Subsequently the species was included in the genus *Shigella*, since it had been reported nonmotile by Hadley. Now that its true reactions are known, it is evident that the species never should have been removed from the genus *Eberthella*. Meanwhile, that genus has been left without a type species by the recognition of the fact that *E. typhosa* really belongs in the genus *Salmonella*. In theory, this destroys the genus *Eberthella* as a taxonomic entity. However, there is need for a group designation for these organisms which have in the past been called *Eberthella* species, and the proposed Bacteriological Code does not specifically forbid changing of the type species of a group essentially subgeneric in rank. It is therefore proposed that the genus *Eberthella* be retained as a useful and necessary group, and that *Eberthella rettgeri* (Hadley), the best known and most widely distributed of the species assigned to the genus, be recognized as the type species.

²³ Buchanan, R. E., *J. Bact.*, 1918, 3, 27.

of broth medium containing respectively 0.15 molar concentrations of sucrose, raffinose, or glucose, the base of each medium was tryptose peptone 1%, anhydrous sodium phosphate 0.2%, and sodium chloride 0.5%. After incubation for 10 days at 23°C, the cultures were centrifuged at moderate speed (1500 rpm) for 40 minutes and the supernatant fluids utilized as the test materials.

The presence of levan was determined by analyzing the fluids for polyfructoside content. The method, described previously in detail³ consisted essentially of repeated precipitation with alcohol, hydrolysis of the final precipitates in 0.8 N HCl for 15 minutes at 60°C and analysis of the hydrolysate for fructose by the Roe⁶ procedure.

The serological procedure consisted of testing a series of dilutions of the neutralized culture fluids against 3 kinds of antisera which were known to react with high dilutions (1:500,000 to 1:2 million) of the previously studied purified levans² viz, antiserum of sucrose-grown bacillus N9, antiserum of sucrose-grown *S. salivarius* S20B, and a selected sample (called lot 1) of type 20 antipneumococcus. The use of the 3 kinds of levan-reactive sera made the comparison of the levans in the culture fluids with the previously studied purified levans more complete than would be furnished by tests with a single serum. Although the type 20 serum reacted with dextrans as well as with levans, this complication was controlled by including in all of the tests another sample (called lot 2) of type 20 and a type 2 antipneumococcus serum, both of which were known to be as highly reactive with dextrans as lot 1 of type 20 but entirely non-reactive with levans.

The results are summarized in Table I. In the case of strains listed individually the polyfructoside values are given for both the sucrose and raffinose fluids, in the cases where several strains are listed as a group, the data given are the lowest and the highest values obtained for either the sucrose or raffinose fluids. The serological data for the

sucrose and raffinose fluids are combined because no significant difference was observed between them.

Results The chief point in the data (Table I) is that the sucrose and raffinose culture fluids of *B. subtilis*, *B. panis*, *Bacillus* strains A to E, and 3 non-sporulating Gram-positive rods, which were proved to contain relatively abundant amounts of levan by the polyfructoside analyses reacted in high dilutions with the same 3 kinds of antiserum with which the previously studied purified levans² had reacted. In contrast, the glucose cultures of the same bacteria contained no levan and gave no reactions with the levan-reactive antisera obtained by immunization with *S. salivarius* and with type 20 pneumococci. Slight reactions did occur with the antibacillus serum but these can be dismissed as due to some minor antigen. The chemical proof for the presence of levan in the reactive fluids, the substrate specificity, the serological likeness to the previously studied purified levans and other factors² make it reasonable to accept the levan nature of the products responsible for the reactions between the anti-levan sera and the sucrose and raffinose cultures of the bacilli. The negative reactions of these cultures with the dextran-reactive type 2 and lot 2 of type 20 antiserum serve as a control on the levan reactions obtained from the selected (lot 1) type 20 antiserum. The absence of detectable dextran in the sucrose cultures of the levan-forming bacilli is of some independent interest since levan and dextran occur together in sucrose cultures of certain streptococci.^{3,4}

The evidence for the serological similarity of the levans of the 7 sporulating and 3 non-sporulating Gram-positive bacilli to each other and to the levans of the previously

‡ The serological reactivity of the levan containing sucrose and raffinose cultures of the bacilli was destroyed by 15 minutes exposure at 55°C in 0.2 N HCl, which was known² to destroy the serological reactivity of the previously studied purified levans. This proof of the lability of the serological properties of the fluids of the bacilli is important because a high degree of susceptibility to mild acid hydrolysis is a well known chemical characteristic of levans.

TABLE I
Chemical and Serological Tests Upon Sucrose, Raffinose, and Glucose Culture Fluids

Kind of bacteria	Sucrose and raffinose cultures									
	Polyfructose content mg %			Precipitation with antisera*				Glucose cultures		
	mg %	Bacillus N9	Strep salivarius	Type 20 pneumococci		Type 2 pneumococcus	Polyfructose content mg %	Precipitation with antisera	Bacillus N9	All others
				lot 1	lot 2					
<i>B. subtilis</i>	750, 500	3000	3000	3000	0	0	0	0	10	0
<i>B. parvus</i>	300, 300	3000	3000	3000	0	0	0	0	10	0
<i>Bacillus</i> (strains A, B, C, D, E)	120 to 1000	>1000	>1000	>1000	0	0	0	0	10	0
Gram positive non spore forming rods (3 strains)	160 to 400	>1000	>1000	>1000	0	0	0	0	0	0
Controls										
<i>Bacillus</i> N9	1500, 1300	10,000	10,000	10,000	0	0	0	0	10	0
<i>S. salinarum</i> S20B	160, 90	3000	3000	3000	0	0	0	0	0	0
<i>Bacillus</i> (7 non levan forming strains)	0	0 or 10	0	0	0	0	0	0 or 10	0	0
<i>L. mesenteroides</i> B	0	0	0	0	0	0	0	0	0	0
sucrose cultures	0	0	0	50,000	50,000	50,000	0	0	0	0
raffinose cultures	0	0	0	0	0	0	0	0	0	0

* Figures indicate highest dilution of the fluid that give precipitation, 0 indicates no precipitation with 1:10 or higher dilutions

† Lot 1 of type 20 pneumococcus serum was known from previous tests to react with high dilutions of purified levans as well as with high dilutions of purified dextrans, lot 2 was as highly reactive with dextrans as was lot 1, but did not react at all with levans

flour proteins in the diet 12 animals made an average gain per animal of 19.3 g and the protein efficiency on this level of intake was 0.72. On the other hand, 30 animals on 5.8% proteins in polished rice made an average gain per animal of 74 g and the efficiency of the rice proteins was 1.86. In other words, on the same plane of protein intake, the animals on the proteins in polished rice gained almost 4 times as much as those on the proteins in enriched wheat flour, also the efficiency of the proteins in polished rice was 158% greater than those in the proteins of the wheat flour.[†]

[†] Since this manuscript was completed a communication was received from Dr. D. B. Jones of the Food and Nutrition Division of the Department of Human Nutrition, Washington, D. C. in

which he states that my results are essentially in agreement with those in progress in his laboratory.

Sure B. *J. Nutr.* 1941 **21**, 455.

15316

Potentiating and Pressor Action of Some N-Substituted Hexylamines

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Since Frolich and Loewi first observed the potentiation of epinephrine by cocaine in 1910, many other compounds have been found which also increase the pressor response of epinephrine. Gaddum and Kwiatkowski¹ stated that the effect of epinephrine was increased by low but inhibited by high concentrations of ephedrine. They suggested that this might be due to the inhibition of amine-oxidase by ephedrine thus preventing the destruction of epinephrine. Lawrence, Morton and Tainter² reported that cocaine, as well as ephedrine and tyramine, blocks the ferment which inactivates epinephrine. Jang³ studied the effect of 10 sympathomimetic amines on the response of epinephrine

and adrenergic stimulation and reported that ephedrine, benzedrine, propadrine, tyramine, cobefrine, sympatol and adrenalone possess the property of sensitizing in small doses and antagonizing in larger doses, the response of epinephrine or adrenergic stimulation on some or all of the preparations used in their study. Munch and Hartung⁴ found the phenylpropanolamine skeleton essential, in their series of arylpropanolamines, for the potentiation of epinephrine. Graham,⁵ in an investigation of a series of amines, found that derivatives of phenylethylamine are not as potent as derivatives of phenylisopropylamine in increasing the effects of epinephrine on the cardiovascular system. Jang,⁶ in a report

¹ Gaddum, J. H., and Kwiatkowski, H. *J. Physiol.* 1938 **94**, 87.

² Lawrence, W. S., Morton, M. D., and Tainter, M. L. *J. Pharmacol.* 1942, **75**, 219.

³ Jang, C. S., *ibid.*, 1940 **70**, 347.

⁴ Munch, J. C., and Hartung, W. H., *J. Am. Pharm. Assn.* 1930, **19**, 356.

⁵ Graham, J. D. P., *Quart. J. Pharm. and Pharmacol.* 1944, **17**, 19.

⁶ Jang, C. S., *J. Pharmacol.* 1941 **71**, 87.

studied^{2,3} bacillus and *S. salivarius* is based upon their likeness in capacities to react with antisera obtained by immunization with 3 phylogenetically different bacteria (bacillus, streptococcus, pneumococcus), which is considerably more significant than a likeness in capacity to react with a single antiserum. The high degree of serological similarity found for all the levans studied indicates that similar serological properties are possessed by a large proportion, if not all, of the different members of this class of bacterial polysaccharide. However, since differences in molecular size and detailed chemical structure may occur among them, the levans of a larger number of bacteria will have to be studied before definite conclusions are made on the serological

properties of the entire class.

Summary Levans formed in sucrose and raffinose broth by *B. subtilis*, *B. pams*, 5 other strains of *Bacillus*, and 3 strains of non-sporulating Gram-positive rod-shaped bacteria possessed serological properties similar to those previously described for the levans of several strains of *S. salivarius* and of one strain of *Bacillus*. This evidence extends the serological information on the levan class of bacterial polysaccharide and gives a better basis for the application of serological procedures to the detection of bacterial levans both in laboratory culture fluids and in the various materials in which they occur in nature.

15315 P

Relative Efficiency of the Proteins in Polished Rice and in Enriched Wheat Flour.*

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While a review of the literature discloses that on the same protein intake (5% level) the proteins in whole rice are superior to those in corn and oats,¹ no information was found on the relative biological values of the proteins in polished rice and milled wheat flour, fed on the same protein intake.

During the past year and a half, while engaged in studies on the influence on growth and protein utilization of additions of small amounts of cultured food yeasts to the proteins of cereal flours and cereal grains, it was observed that, on the same protein level in the ration, the proteins in polished rice are far superior to those in enriched white flour. Polished rice fed at an 89% level and enriched wheat flour fed at 57% plane of

intake furnished the same 5.8% protein in the ration. The rations contained 2% cellu flour for roughage, 2% cod liver oil and 3% wheat germ oil as sources of fat-soluble vitamins, and a complete salt mixture.² Cerelese (dextrose) constituted the rest of the rations. An abundance of the vitamin B complex was furnished daily to each animal separately from the ration. The composition of the vitamin B complex mixture was as follows: 100 µg thiamin, 100 µg riboflavin, 100 µg pyridoxine, 600 µg calcium pantothenate, 100 µg nicotinic acid, 12 mg choline chloride, 12 mg p-aminobenzoic acid, and 3 mg inositol. The protein efficiency is expressed as gains in weight per gram of protein intake. The experimental period was 10 weeks.

Twelve animals on 9% enriched patent wheat flour proteins showed an average gain per animal of 42.2 g and the proteins had an efficiency of 0.88, while on 5.8% wheat

* Research paper No. 809 Journal Series, University of Arkansas. Published with the approval of the Director of the Arkansas Agricultural Experiment Station.

¹ Mitchell, H. H., *J. Biol. Chem.*, 1924, **58**, 905.

² Sure, B., *J. Nutr.*, 1941, **22**, 499.

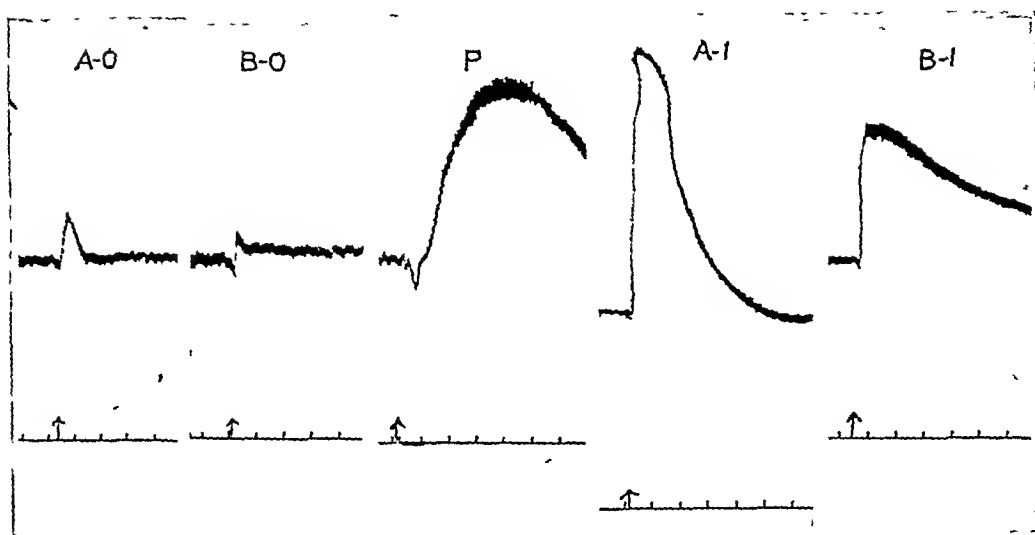


Fig 1

Potentiation of pressor amines by other amines Carotid blood pressure of anesthetized dog All injections were made into the exposed femoral vein

A 0 N methyl beta cyclohexylethylamine HCl 0.10 mg/kg before potentiation

A 1 Same after potentiation

B 0 Ephedrine HCl 0.05 mg/kg before potentiation

B 1 Same after potentiation

P Hexyl gamma cyclohexylpropylamine HCl 1.00 mg/kg

amines An investigation of the pressor and potentiating actions of these amines was carried out and the results are reported here

Experimental Dogs anesthetized with sodium pentobarbital and atropinized were used as the experimental animals. Blood pressure was recorded from the carotid artery. All injections were made into the exposed femoral vein.

The compounds studied are listed in Table I along with the results indicating their pressor activity. They were dissolved in water or 50% ethanol, as indicated in the table, to make a 1% solution of the hydrochloride salt except in the case of n-hexylamine in which case an aqueous solution of the base was used.

The most active pressor compound in the series is No. 185 (N-methyl-hexylamine). Other compounds which show marked pressor activity are Nos. 445, 477, 489, 490, 491, and 492. Compound No. 491 is approximately one-half as active as N-methyl-hexylamine on blood pressure. The pressor activity of compound No. 491 is greater than that of its analog, N-methyl-gamma-cyclo-

hexylpropylamine, the pressor action of which was described by Lands, Lewis and Nash.¹¹ In general, the cyclohexylalkylamines are the most active in this series.

Compounds of the series with groups larger than methyl substituted in the amine group of hexylamine produce a diphasic type of blood pressure response which is characterized by a sharp transient fall followed by a more sustained rise. An example of this is shown in Fig. 1. We believe the fall results from a non-specific depressor action and may be due to a direct relaxing action of the compound on the musculature of the vessels. Tachyphylaxis was observed whenever repeated injections of these compounds were made.

The potentiating effect of a few sympathomimetic amines was determined by comparing the pressor response of the sympathomimetic compound before and after an injection of one of the various hexylamine derivatives. The resulting potentiation is

¹¹ Lands, A. M., Lewis, J. R., and Nash, V. L., *J. Pharmacol.*, 1945, 83, 253.

TABLE I
Effect on Blood Pressure of N Substituted Hexylamines, $C_6H_{13}NH R$

Compound No	R	No of animals	Mean of doses (mg/kg)	Mean of change in blood pressure (mm Hg)
	H	4	0.4	+30
185	Methyl	5	(0.3 0.5) †	(-16, +50) †
			0.5	+55
443	Butyl	5	(0.2 1.0)	(-24, +90)
			0.9	-1
444	Amyl	4	(0.5 1.0)	(-10, +18)
			1.0	+13
445	Hexyl	12	0.9	(-20, +46)
			(0.5 1.0)	+19
354*	Heptyl	5	0.9	(-50, +100)
			(0.5 1.0)	-6
446*	Octyl	4	1.0	(-25, +27)
				+10
474	Phenylmethyl (Benzyl)	4	1.0	(-20, +36)
				0
475	beta Phenylethyl	8	1.0	(-20, +36)
				-2
476	gamma Phenylpropyl	4	1.0	(-30, +30)
				0
477	delta Phenylbutyl	4	1.0	(-20, +20)
				+38
478*	epsilon Phenylamyl	4	1.0	(-10, +60)
				+7
479*	zeta Phenylhexyl	5	1.0	(-15, +25)
				-4
343	Cyclohexyl	8	1.0	(-40, +75)
				0
489	Cyclohexylmethyl	6	0.9	(-33, +16)
			0.5	+30
490*	beta Cyclohexylethyl	9	(0.5 1.0)	(-25, +75)
			1.0	+35
491*	gamma Cyclohexylpropyl	5	(0.5 3.0)	(0, +145)
			1.0	+56
492*	delta Cyclohexylbutyl	4	1.0	(-22, +90)
				+23
493*	epsilon Cyclohexylamyl	3	1.0	(0, +42)
				-10
494*	zeta Cyclohexylhexyl	9	1.0	(-75, +30)
				-7
				(-40, +18)

* Compounds dissolved in 50% ethanol Others dissolved in water

† Range of values

on the potentiation of epinephrine by ergotovine, stated that no chemical group is specific for the synergistic or antagonistic action towards epinephrine except that which is essential for sympathomimetic action, and according to Barger and Dale⁷ this is an aliphatic amine group. Of the aliphatic amines studied by these workers, n-hexylamine was found to have the greatest vaso-pressor action.

Blicke and Monroe,⁸ Blicke and Zienty⁹

and Blicke and Data¹⁰ have synthesized, and made available to this laboratory, a series of compounds which can be considered as derivatives of n-hexylamine. While carrying out a routine investigation of these substances, it was noted that some of the amines potentiated the vaso-pressor action of epinephrine and of various other sympathomimetic

⁸ Blicke, F. F., and Monroe, E. *J. Am. Chem. Soc.*, 1939, **61**, 91

⁹ Blicke, F. F., and Zienty, F. B., *ibid.*, 1939, **61**, 771

¹⁰ Blicke, F. F., and Data, John B., *Dissertation*, University of Michigan, 1941

⁷ Barger, G., and Dale, H. H., *J. Physiol.*, 1910, **41**, 19

TABLE III
 Acute Toxicity of Some N-Substituted Hexylamines

Compound No	Approximate LD 50, mg/kg	Dose (mg/kg), No of deaths/No injected
n Hexylamine	25	15, 0/2, 25, 5/12, 50 2/2
445	30	15 1/2 25, 2/12 35, 5/5
474	70	40, 0/2 60 1/10, 70, 8/10, 80, 7/10, 100, 4/5 120, 2/2
479	25	15, 2/2 25 7/12 35, 3/5 50, 10/10
489	50	35 0/10 50 7/10 75, 2/2
494	<15	15 10/12, 25 12/12

hexylamine), was found to be no more toxic than the primary compound, n-hexylamine

Discussion Barger and Dale,⁷ in their study of the relationship between chemical structure and sympathomimetic activity, found n-hexylamine to be the aliphatic amine having maximum pressor activity. This activity was decreased when the aliphatic chain was lengthened or when there was a substitution in the amino group. Unfortunately we did not have available the N-ethyl- and N-propyl-hexylamines to complete our series. N-methyl-hexylamine was found to have the greatest pressor action, di-hexylamine is the next most active amine in the aliphatic series. In view of the previous work, it was somewhat surprising to find pressor activity in an aliphatic secondary amine of this size. The marked pressor response of some of the other compounds such as No. 491, which was most active of the compounds containing a ring, was also not expected inasmuch as it contains a 6-carbon chain substituted on the nitrogen in addition to a 3-carbon chain between the ring and the nitrogen. On the basis of the results obtained with these compounds, it would appear that the pressor activity is primarily a result of the action of the hexylamine portion of the molecule but is modified quantitatively by the other group substituted on the nitrogen.

Since many types of compounds have been reported to potentiate the pressor response of epinephrine, there does not seem to be any chemical group specific for this action. Gaddum and Kwiatkowski¹ have attempted to explain the potentiation of epinephrine by ephedrine with the theory that ephedrine inhibits amine oxidase thus preventing the destruction of epinephrine. This theory of

substrate competition has also been applied to explain the pressor action of ephedrine. Ephedrine has been shown not to be destroyed by this enzyme by Blaschko *et al*.¹² The same theory has been applied to the sensitization of epinephrine by cocaine since Lawrence, Morton and Tainter² reported that cocaine inhibits amine-oxidase. The pressor response of ephedrine and many other sympathomimetic compounds, including beta-phenylethylamine (Tainter¹³) is diminished by cocaine whereas they are potentiated by compounds in our series. This would indicate that there is more than one mechanism involved in the potentiation phenomenon.

Jang³ found that cobefrine, which is not attacked by amine-oxidase, was potentiated by ephedrine. We found beta-phenylethylamine, which is destroyed by amine-oxidase and ephedrine which is not destroyed by the enzyme (Beyer and Morrison¹⁴), both potentiated by hexylamine derivatives. If the pressor action of ephedrine is due to its inhibition of amine-oxidase, then we could not expect it to be potentiated by other sympathomimetic amines whose mechanism of action has been suggested to be the same as ephedrine.

There are a number of enzyme systems which have been recently reviewed by Bever and Morrison¹⁴ that are capable of inactivating sympathomimetic amines under certain conditions. When one compound increases the action of several sympathomimetic agents

1. Blaschko, H., Richter, D., and Schlossmann, H., *Biochem. J.*, 1937, **31**, 2187.

13. Tainter, M. L., *Arch. intern. Pharmacodyn.*, 1933, **46**, 192.

14. Beyer, K. H., and Morrison, H. S., *Ind. and Eng. Chem.*, 1945, **37**, 143.

TABLE II
Effect of N Substituted Hexylamines on Pressor Action of Sympathomimetic Amines

Potentiating compound	Sympathomimetic Amine															
	Epinephrine				N methyl beta cyclohexylethylamine				beta Phenyl ethylamine				Ephedrine			
	A	B	C	D	A	B	C	D	A	B	C	D	A	B	C	D
185*	4	8	39	-8	4	25	37	-5	—	—	—	—	—	—	—	—
443	3	67	32	+2	2	1	15	+10	2	075	22	+17	1	1	14	+2
444	3	67	35	+6	1	1	5	+37	3	067	21	+21	1	1	12	+15
445	9	89	30	+4	9	12	20	+30	3	084	19	+40	1	1	12	+23
354	3	86	31	+5	3	1	25	+38	3	067	21	+45	1	05	18	+10
446	2	10	18	+15	3	1	24	+32	2	067	24	+35	1	05	12	+28
474	3	86	27	+4	2	1	13	0	3	067	31	+2	1	05	12	0
475	6	11	32	+1	3	13	31	+8	2	095	34	-2	2	075	14	0
476	2	5	25	0	3	1	20	+32	1	05	32	0	2	075	14	+2
477	2	5	28	+4	3	13	24	+48	1	05	32	+12	2	075	16	+17
478	2	10	23	+5	3	1	23	+26	1	05	17	+18	1	05	12	+4
479	4	9	35	+3	3	1	22	+24	2	1	24	+22	2	075	16	+8
343	5	11	32	-1	5	12	17	+3	2	075	19	+14	1	05	10	+8
489	2	5	39	-4	4	18	27	+16	2	05	20	+6	1	05	12	+3
490	7	18	41	+12	5	14	32	+31	1	05	12	+20	1	05	8	+11
491	4	11	40	+15	4	15	28	+42	1	05	20	+7	2	05	13	+32
492	3	67	38	0	2	15	28	+33	3	05	21	+27	2	05	13	+12
493	—	—	—	—	1	2	19	+41	1	05	11	+20	1	05	6	+6
494	5	11	34	+20	8	15	23	+33	3	05	22	+22	2	05	11	+6

* Dose of potentiating compound was 1 mg/kg except No. 185 which was 5 mg/kg

† Column A—No. of animals used

Column B—Me in dose in mg/kg except epinephrine which is in gamma/kg

Column C—Average mm rise in blood pressure before the potentiating compound

Column D—Average change in column C after the potentiating compound

given in Table II, and an example is shown in Fig. 1. Since the data represents only a small number of observations in each case the results cannot be compared quantitatively.

The results obtained with epinephrine were much more variable than those obtained with the other sympathomimetic compounds. The inclusion of experiments in which there was no potentiation or even a decrease in the response reduces the positive value of the averages. Compound No. 185 did not produce any sensitization of the pressor responses in the few experiments in which it was used. A sufficient quantity of this compound was not available to carry out further tests. N-methyl-beta-cyclohexylethylamine was not potentiated by No. 474. All other compounds caused an increase in the pressor response of this sympathomimetic amine. The pressor response of beta-phenylethylamine was sensitized by all compounds in the series except Nos. 475 and 476. No specific conclusions can be made concerning the potentiation of ephedrine because of the small number of observations. However, the re-

sults obtained appear to parallel those of beta-phenylethylamine.

The pressor activity of the various compounds bears no relationship to their potentiating effect. The duration of the sensitization was found to vary in different experiments from a few minutes to as long as 2 hours. However, in every case it was a reversible phenomenon and the increased pressor responses of the sympathomimetic amines returned to normal, as determined by repeating the injection at various intervals of time. Other sympathomimetic compounds which were observed to be potentiated are n-hexylamine, tyramine, amphetamine, Sympatol and Privine.

Acute toxicity of a few of these compounds was determined by intraperitoneal injection in albino mice. The results are given in Table III. With the substituted hexylamines, those containing a cyclohexyl ring are more toxic than those containing a phenyl ring. Toxicity is increased by increasing the length of the chain between the ring and the nitrogen. The secondary amine, No. 445 (di-n-

TABLE I

Summary of Experiments Upon Protective Action of Various Agents Against Parenchymal Necrosis in Liver and of Lipotropic Action

Supplement to basic diet avg per animal		No rats	Avg % wt lost	Hepatic cell necrosis series		Fatty infiltration of liver			
				No	%	Moderate* series		Severest series	
						No	%	No	%
Basic diet only		48	17.7	6	12.5	13	27	15	31.2
Choline	41.8 mg	20	8.6	0		0		0	
Choline	20	20	3.2	0		5	25	0	
Cystine	32.3 }								
Methionine	40	18	2.3	0		5	27.8	1	5.5
Thioglycolic acid	24.6	25	3.0	1	4.0	3	12	10	4.0
Glycolic acid	23.3	20	1.4	1	5.0	7	35	10	5.0
Thiomalic acid	31.75	20	18.6	2	10.0	0		19	9.5
Malic acid	35.8	19	13.0	2	10.5	5	26.3	9	47.5
Thiolactic acid	28.45	19	15.0	3	15.8	4	21.0	11	58.0
Total No rats		209							

* + + fatty infiltration (see text)

† + + + + or + + + + + fatty infiltration (see text)

Other —SH compounds were also tested but a close correlation between the presence of —SH and protection against hepatic damage was not here observed. Methionine in large doses did not afford protection. The following studies were conducted to determine if certain —SH and other compounds might afford protection against hepatic necrosis of dietary deficiency in the rat and also to observe if such compounds might possess lipotropic action.

Methods. White rats of inbred strain weighing at least 150 g were maintained upon the following diet for 70 days:

	%
Casein (vitamin free)	8
Crisco	38
Glucose	48
Cod liver oil	2
Salt mixture ³	4

Vitamins added to afford each animal approximately the following daily intake: Thiamine hydrochloride, 75 µg, pantothenic acid, 100 µg, riboflavin, 40 µg, pyridoxine, 65 µg, niacinamide, 5 mg. (These were supplied in vitamin B complex-oral, Lederle Lab Inc.)

This diet has been shown⁴ to result in

³ Hubbell, R. B., et al., *Am J Nutrition*, 1937, 14, 273.

⁴ Gyorgy, P., and Goldblatt, H., *J Exp Med*, 1942, 75, 355.

varying degrees of fatty infiltration of the liver, cell necrosis and if maintained for sufficient periods, cirrhosis.

The animals were kept in groups of 8 to 10 in a cage. The daily individual intake of food was not measured but the animals were weighed at weekly intervals and the average of each cage recorded. This served as the indicator of food ingested.

Supplements consisting of the various agents to be tested for protective action on the liver were added to the several portions of the basic diet as indicated in Table I. The quantities of sulphur in the —SH compounds added were equivalent to the S in 40 mg of methionine.

In a few instances the animals died before the expiration of the experiment; the remainder were killed at the termination of the period stated and the livers were fixed in 5% formaldehyde embedded in paraffin and sections stained with hematoxylin and eosin, and in some instances by Mallory's connective tissue stain. Frozen sections for staining with scarlet red were also made in a number of instances.

The features noted were degree of necrosis (+ to + + + +), degree of fatty infiltration (a few scattered vacuolated cells, +, larger groups of vacuolated cells, + +, wide zones of vacuolated cells, + + +, practically every cell vacuolated + + + +). The fact that

which are probably inactivated by different types of enzymes, the theory of substrate competition will not explain the mechanism, unless the potentiating compound were capable of reacting with the various enzymes. This does not seem probable in view of the degree of specificity exhibited by enzyme systems. The effector cell may be sensitized by these potentiators. This raises a fundamental question with regard to the mechanism of action of the various sympathomimetic agents. A completely satisfactory answer has not yet been found. However, it does not seem unlikely that compounds containing long aliphatic chains might alter permeability by surface action and thereby increase the sensitivity of the cell to other compounds.

Summary 1 A series of 19 *N*-substituted hexylamines have been studied for their effect on blood pressure and on potentiation of the pressor response of some sympathomimetic compounds. 2 *N*-methyl-hexylamine was found to have the greatest pressor action with di-hexylamine next most active in the aliphatic series. 3 *N*-(gamma-cyclohexylpropyl)-hexylamine has the great-

est pressor action of those compounds containing a ring. In general, compounds containing a cyclohexyl ring were more active than those containing a phenyl ring or with only aliphatic groups. 4 Many of the compounds in this series have been shown to potentiate the pressor response of epinephrine, beta-phenylethylamine, *N*-methyl-beta-cyclohexylethylamine, ephedrine and some other sympathomimetic agents. The potentiation of epinephrine was more variable than that of the other sympathomimetic compounds. 5 The possible mechanisms of the potentiation phenomenon are discussed. The amine-oxidase theory of potentiation does not appear to apply to the compounds in this series. 6 Acute toxicity tests indicate that those compounds containing a cyclohexyl ring are more toxic than those with a phenyl ring. Increasing the length of the chain between the ring and the nitrogen increases the toxicity. Di-*n*-hexylamine is no more toxic than *n*-hexylamine.

The author is indebted to Mrs. Kathryn Z. Hooper for assistance in carrying out some of the experiments and to Miss V. Lorraine Nash for the determination of acute toxicity.

15317

Protective Action of Certain Compounds Against Dietary Hepatic Damage in the Rat *

JOHN GREEN AND A. BRUNSCHWIG

From the Department of Surgery, University of Chicago

In a previous communication¹ experimental studies were reported to show that the protective action of protein (casein) and methionine against chloroform hepatic damage in the dog could be accounted for by the presence of —SH since thioglycolic acid conferred similar protection to the above and glycolic acid, aspartic acid, glutamic acid and sodium

thiosulphate did not afford protection. This conclusion was further strengthened by hitherto unpublished experiments in which thiomalic and thiolactic acids conferred protection but malic and lactic acids failed to afford protection. In a second report² it was shown that thioglycolic acid conferred protection in the rat against acute hepatic damage by carbontetrachloride but glycolic acid conferred almost an equal degree of protection.

* These studies were conducted under the Charles and Mary F. S. Worcester Fund. The University of Chicago.

¹ Brunschwig, A., *et al*, *Arch. Path.*, 1945, 40, 81.

² Brunschwig, A., Johnston, C., and Nichols, S., *Proc. Soc. Exp. Biol. and Med.*, 1945, 60, 388.

TABLE I
Urinary Excretions of Porphyrins

Dates 1941	Vol cc	ug pooled coproporphyrin	Proto porphyrin	Vol cc	ug pooled copro porphyrin	Proto porphyrin
5/21 to 23	40	7	none	57	7	none
5/23 to 26	80	11	"	84	7	"
5/26 to 28	51	11	"	63	7	"
5/28 to 30	56	7	"	71	5	"
5/30 to 6/2	84	11	"	88	7	"
Injection	solvent only—N/50 NaOH			10 ug/kg protoporphyrin in N/50 NaOH		
6/2 to 4	74	7	none	70	7	none
6/4 to 6	80	11	"	85	7	"
6/6 to 9	90	24	"	94	21	"
6/9 to 11	60	16	"	79	11	"

It has frequently been assumed that coproporphyrin might be derived from protoporphyrin in the body. In perfusion experiments, van den Bergh, Grotepass, and Revers⁵ found that liver converted protoporphyrin into coproporphyrin, but Watson⁶ and his co-workers were unable to confirm this. The work here reported was designed to bring evidence on this question and also to test the effectiveness of protoporphyrin in photosensitization.

Experimental Protoporphyrin was prepared by the following method suggested by Dr E. H. Maechling.⁷

To human clotted blood, was added an equal volume of concentrated HCl and the mixture was heated over a steambath for an hour till all clots were completely broken up. The dark red mixture showed absorption bands at 667-640 m μ , 602-593 m μ , 588-546 m μ . This was cooled and distilled water added—3 parts water to one part blood mixture. The diluted mixture was twice

extracted with 1½ volumes chloroform. The acid chloroform extract was dichroic (greenish-pink), had a red fluorescence and showed absorption bands at 612-604 m μ , 587-583 m μ , and 568-554 m μ . The emulsion formed at this point was broken up by shifting the mixture from one separatory funnel to another and allowing it to separate for 20 to 40 minutes. The chloroform solution was washed with portions of water until the washings were neutral to litmus. The neutral chloroform extract showed absorption at 634-628 m μ , 590-577 m μ , and 547-536 m μ . As the acid was removed, the chloroform solution turned from a dichroic greenish-red to a red color. The neutral chloroform solution was extracted with 1/6 volume N/20 NaOH. The dark brown alkaline solution had absorption bands at 638-628 m μ , 585-573 m μ , and 557-543 m μ . Twenty-five cc glacial acetic acid were added. This was then extracted with 200 cc ether. The acid layer was washed with distilled water until washings were neutral to Congo Red. The ethereal solution was then filtered. The pink neutral ethereal extract had an intense red fluorescence and strong absorption bands at 637-630 m μ , 606-581 m μ , 577-574 m μ , 542-531 m μ , 512-499 m μ . The ether was evaporated off the resulting amorphous residue was virtually pure protoporphyrin, as indicated by solubility and spectral characteristics.

Various dilutions of the protoporphyrin in 16 cc ether were made and studied spectroscopically in a 200 mm polariscope tube. The end-points of spectral band detectability

⁴ a Rimington, C, *Onderstepoort J Vet Sci and Animals Ind*, 1936, 7, 567, b Dobriner, K, *J Biol Chem*, 1937, 120, 115, Borst, M u, and Komgsdorffer, H, *Untersuehungen uher Porphyrie mit besonderer Beruecksichtigung den Porphyria congenita*, Leipzig, S Hirzel, 1929, d Dobriner, K, and Rhoads, C P, *Phys Rev*, 1940, 20, 416.

⁵ Van den Bergh, A A H, Grotepass, W u, and Revers, F E, *Klin Wchnschr*, 1932, 11, 534.

⁶ a Watson, C J, Pass, I J, and Schwartz, S, *J Biol Chem*, 1941, 139, 583, b Salzburg, P, and Watson, C J, *J Biol Chem*, 1941, 139, 593.

⁷ Personal communication.

these vacuoles represented fat was controlled by appropriate stains since in marked protein depletion the cytoplasm of hepatic cells often appears vacuolated but does not contain fat). The presence of cirrhosis was also sought for but this was not frequently encountered due in all probability to the duration of the experiments. It was observed only in 3 instances, one in each of the groups receiving glycollic, thiolactic and thiomalic acids respectively.

The results are summarized in Table I.

Discussion From the table it is noted that cellular necrosis occurred in 12.5% of the control series and moderate to severe fatty infiltration was present in 58%. Choline afforded absolute protection against necrosis and fatty infiltration. Choline in reduced quantity plus cystine protected against necrosis and also exhibited lipotropic action but not as well as larger doses of choline alone. Methionine exhibited approximately the same effects as choline plus cystine. Thioglycollic and glycollic acids afforded appreciable but not absolute protection against necrosis and *did not exhibit lipotropic action*. Indeed glycollic acid afforded

a higher incidence of fatty infiltration than in the control series. Thiomalic, malic and thiolactic acids afforded no protection against necrosis and resulted in a higher incidence of fatty infiltration than in the control series.

The results with thioglycollic and glycollic acids are of interest since by their use it would appear that the lipotropic factor and necrosis protecting factor are shown not to be necessarily identical. For further support of this is the fact that methionine, which has been repeatedly shown to be lipotropic, does not protect against the acute hepatic cell damage caused by carbontetrachloride in rats.

The explanation of the protective action of thioglycollic and glycollic acids against acute hepatic cell damage by carbontetrachloride and by dietary deficiency is not apparent.

Summary Both thioglycollic and glycollic acids afford a degree of protection against hepatic cell necrosis due to dietary deficiency in rats. These compounds are not lipotropic. The above studies indicate that in certain dietary deficiencies in rats leading to hepatic fatty infiltration and parenchymal necrosis, the factors responsible for both these changes are not necessarily identical.

15318

Experiments with Protoporphyrin

I ZELIGMAN (Introduced by J. G. Hopkins)

From the Departments of Dermatology and Biochemistry, Columbia University, College of Physicians and Surgeons, and from the Skin and Cancer Unit, New York Post Graduate Medical School and Hospital, Columbia University

It has recently been shown¹ that the chromogen of hemoglobin is synthesized *in vivo* from acetate and glycine, but little is known with certainty of the metabolic origin of the porphyrins of the excreta. It has been variously suggested that these are formed (a) during the breakdown of endogenous hemoglobin to bile pigments,² (b) by con-

version of ingested chlorophyll and hemoglobin³ and (c) by processes involved in the synthetic production of hemoglobin.⁴

¹ a Schreus, H. T., and Currie, C., *Klin Wochenschr*, 1933, 12, 745, b Herold, L., *Arch f Gynak*, 1934, 158, 213.

² a Snipper, J., *Arch f Verdauungskr*, 1919, 25, 230, b Selmann, O., *Z f physiol Chem*, 1926, 153, 225, c Krummeier, H., *Deutsches Arch f Inn Med*, 1924, 145, 257, d Vannotti, A., *Porphyrine und Porphyrinderivate*, Berlin, J Springer, 1937.

¹ a Bloch, K., and Rittenberg, D., *J Biol Chem*, 1945, 159, 45, b Shemin, D., and Rittenberg, D., *J Biol Chem*, 1945, 159, 567.

Summary 1 A simple method for the preparation of protoporphyrin from blood is described 2 White rats were injected subcutaneously with protoporphyrin and were found to excrete greatly increased amounts of protoporphyrin in the feces No urinary protoporphyrin nor increase of urinary coproporphyrin excretion following the injection was noted Much of the injected protoporphyrin remained at the site of injection There was no evidence that the rat is able to convert injected protoporphyrin to coproporphyrin 3 Photosensitization experiments of protoporphyrin-injected white rats to carbon-arc and sunlight were unsuccessful

* Aided by a grant from the National Foundation for Infantile Paralysis, Inc

TABLE II
 Urinary and Fecal Excretion of Porphyrins

Dates 1941	Vol cc	μg urine copro porphyrin	μg stool copro porphyrin	μg stool proto porphyrin	Vol cc	μg urine copro porphyrin	μg stool copro porphyrin	μg stool proto porphyrin
6/13 to 16	80	11	43	41 ?	70	11	61	58 ?
6/16 to 18	170	8	29	147	74	7	47	247
6/18 to 20	65	11	89	454	65	14	33	363
6/20 to 23	105	24	99	517	90	24	55	265
Injection		solvent 10 cc N/50 NaOH/kg				protoporphyrin 10 mg in 10 cc N/50 NaOH/kg		
6/23 to 25	75	8	116	606	45	7	42	1266
6/25 to 27	81	7	89	545	70	11		1764
6/27 to 30	125	34	56	487	110	36	98	942
6/30 to 7/2	105	14	34	337	75	32	78	1184

were observed with 4 γ at 633, 12 γ at 576 $m\mu$, and 20 γ at 606 $m\mu$. It had previously been observed that hematoporphyrin and coproporphyrin were spectroscopically indistinguishable. Their corresponding end-points of detectability were observed with 3 γ at 623 $m\mu$, 7 γ at 567 $m\mu$, and 14 γ at 596 $m\mu$.

Quantitative estimation of the amount of porphyrin with this spectroscopic method was based on Lageder's⁸ method as employed by Kapp and Coburn⁹ and by Maechling.¹⁰ The spectroscopic examination was made with a Bausch and Lomb wave-length spectrometer No. 628. The light source was a (Burn Base Down) Projection bulb—Mazda G E 6V, 18A.

Animal Studies. Eight white rats weighing about 200 g each, were kept, 4 in each of 2 metabolism cages, on vitamin D free¹¹ Rockland rat diet. The pooled urine from each cage was collected every 48-72 hours and the urine extracted for proto- and for coproporphyrin, as follows:

Urine was treated with 3 cc of saturated sodium acetate solution and $\frac{1}{3}$ volume of glacial acetic acid and extracted twice with equal volumes of ether. The combined ethereal solutions were washed twice with

$\frac{1}{3}$ volume dilute sodium acetate solution (approximately 10 cc saturated sodium acetate solution to 600 cc distilled water) and extracted 3 times with 10 cc 5% HCl. The combined acid extracts were neutralized to Congo Red with saturated sodium acetate solution, and 10 cc of glacial acetic acid were added. The solution was extracted twice with equal volumes of ether and the combined ethereal solution was washed twice with dilute sodium acetate solution and shaken 3 times with 10 cc portions of 0.5% HCl. The combined acid solution was washed with an equal volume of chloroform, neutralized to Congo Red with saturated sodium acetate solution, and treated with 2 cc of glacial acetic acid. The acidified solution was extracted twice with 10 cc of ether and the combined ethereal solution containing coproporphyrin examined spectroscopically by the absorption band end-point method described.

The chloroform solution was extracted twice with 10 cc of N/20 NaOH, the combined alkaline solution acidified with glacial acetic acid and extracted twice with 10 cc of ether. The ethereal solutions were combined and examined spectroscopically by the dilution method for protoporphyrin.

After the approximately basal level of coproporphyrin output had been determined, the 4 animals in one cage were injected subcutaneously with protoporphyrin dissolved in N/50 NaOH at a level of 50 mg per kg body weight. The 4 animals in the other cage received an equal subcutaneous injection of the solvent alone. No striking dif-

⁸ Lageder, K, *Arch f Verdauungsler*, 1934, 56, 237.

⁹ Kapp, E M, and Coburn, A F, *Brit J Exp Path*, 1936, 17, 255.

¹⁰ Maechling, E H, *J Lab and Clin Med*, 1941, 26, 1676.

¹¹ Slonetz, C A, *Am J Vet Research*, 1943, 4, 182.

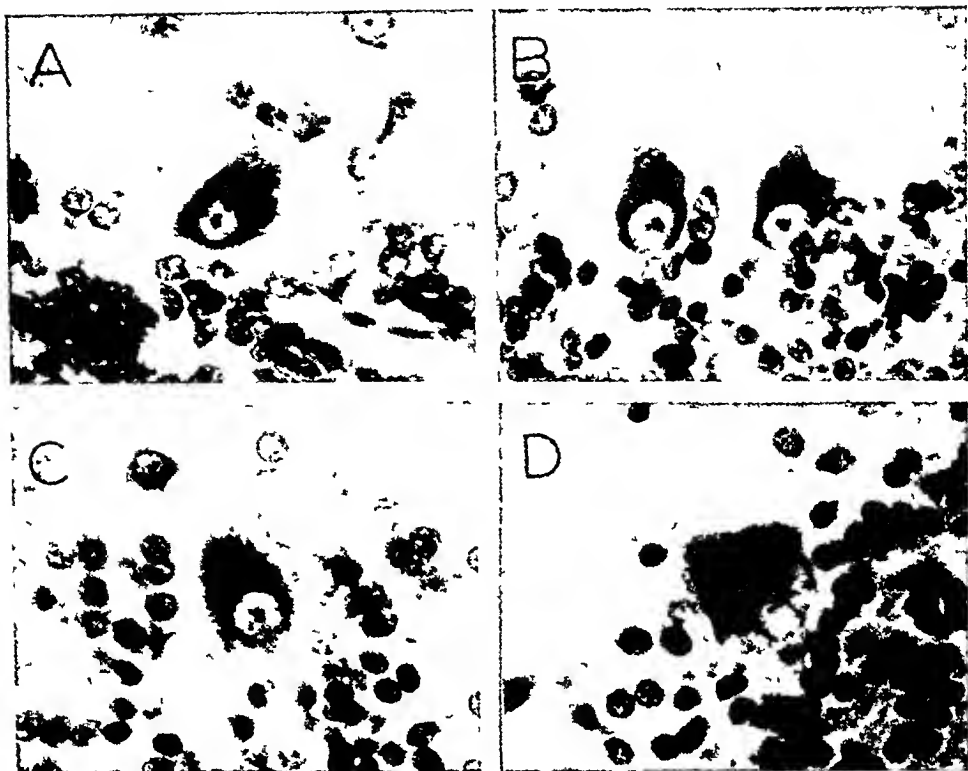


Fig 1

- a Normal Purkinje cell from control preparation
 b Chromatolyzed cells
 c Cell showing clear margin of cytoplasm
 d Cell showing vacuolization of margin $\times 600$

tion developed in a week or 10 days

Immediate and severe chromatolysis produced by irradiation is in marked contrast to the more familiar axon reaction. Changes concomitant with chromatolysis and apparently related to it, the formation of a clear

peripheral zone of cytoplasm and the appearance of vacuoles, appear only as immediate effects. Temporally these are related to certain symptoms which are also fugitive. In several days the whole syndrome is replaced by the well-known degenerative picture

15320

Time Course of Changes in Sensory Cells of Trigeminal Ganglion of the Rabbit Following Irradiation *

ROSALIND NOVICK (Introduced by Berry Campbell)

From the Department of Anatomy, University of Minnesota

Immediate and delayed effects of Roentgen

* Aided by a grant from the National Foundation for Infantile Paralysis, Inc

ray exposure upon neurons may be separated by analysis of the time course of chromatolysis induced. To achieve this end it is essential that single effective doses be employed

TABLE I

Rabbit	Dose	Survival
330	1000 r (220 Kv, $\frac{1}{4}$ mm Cu)	16 hr
331	" "	24 "
332	" "	48 "
333	" "	4 days
334	" "	9 "
335	" "	69 "
347	3000 r	died during exposure
348	" "	" following "
349	" "	18 hr
350	" "	43 "
351	" "	8 days
352	" "	13 "
401	" "	0 hr
402	" "	still alive
403	" "	5½ hr
404	" "	12 hr
405	" "	18 "
406	" "	still alive
407	3500 r (140 Kv, no filter)	0 hr
408	4000 r (220 Kv, $\frac{1}{4}$ mm Cu)	0 "
409	" "	3 "
410	" "	13 "
411	" "	still alive
412	" "	" "
400	" "	" "

these changes has not been made in most of the reported studies. The present investigations were designed to clarify this matter by utilizing single massive exposures which serve as a starting point in relating histological change with elapsed time.

Twenty-five rabbits, 4 to 5 lb in weight, were irradiated in 4 groups of 6 and one singly. With the aid of a special holder, the heads of the animals were held in place in a circle of small radius and simultaneously irradiated. The dosage is listed in Table I.

The changes observed in the treated animals were chromatolysis, vacuolization of the cytoplasm, pyknosis, and loss of cells through neuronophagia. The animals killed immediately following irradiation showed marked lysis of the Nissl material of the cells. This chromatolysis is short-lived, being but slight in No. 349 which was killed at 18 hours and not to be noticed at all in the first member of the 1000 r series. Rabbit No. 408, killed immediately after irradiation shows cells which demonstrate not only complete breaking up of the Nissl bodies into a fine lightly staining powder, but also reveal the appearance at the periphery of the cell of a wide margin of clear cytoplasm. Neighboring cells

show large clear vacuoles occupying this marginal zone and in some instances replacing entirely the unstained cytoplasm. Further studies on the relationship of the chromatolysis, the appearance of the clear margin of cytoplasm, and the vacuolization are being made.

Pyknosis of some cells is seen in the early members of the series and becomes more marked in those killed later. Neuronophagia is not observed where less than 24 hours had elapsed following irradiation. It is severe in all of the later instances.

There is a parallelism between the cellular pathology described above and the observed symptomatic behavior of the animals. Those receiving the heavy doses were observed to be apathetic, somnolent, and even prostrate immediately thereafter. All showed dyspnea and slight or marked exophthalmos. Rapid clearing up of these conditions followed the termination of the irradiation. In one-half hour marked improvement was seen and 12 hours later the survivors showed only slight signs. Conjunctivitis developed during the first 12 hours and lasted as long as 3 or 4 days. Hyperexcitability and excessive saliva-

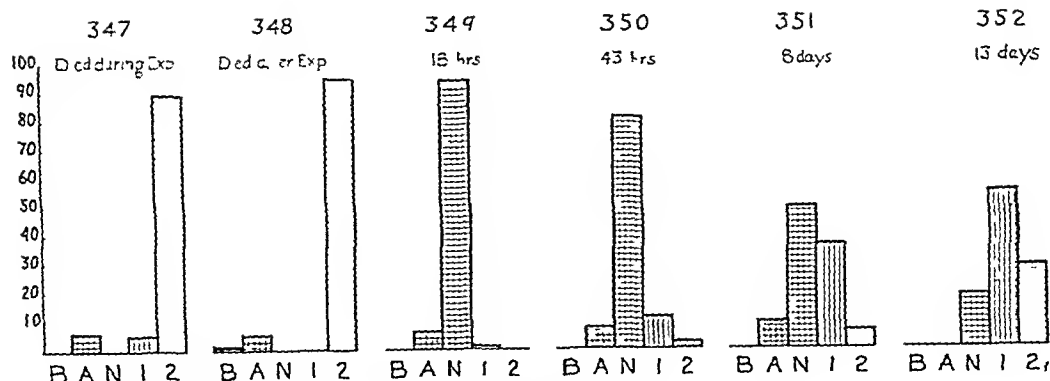


Fig 2

Frequency polygons illustrating differential cell counts of irradiated ganglia

ported in the foregoing paper² For histological methods and experimental conditions reference may be made to that paper

The trigeminal ganglia of 20 irradiated animals, which had received single doses of 1000-4000 Roentgen units to the head were examined to determine the changes in Nissl pattern as a result of irradiation In the second series of rabbits, animals 447-452, which had received 3000 Roentgen units, 100 successive cells from each ganglion were counted and classified into the groups described below Although all the neurons were observed to undergo comparable changes, because of the difficulty of evaluating severity of chromatolysis in small cells, counts were made only of cells 33 μ in diameter or larger

The establishment of a chromatolytic index for the sensory cells of the trigeminal ganglion required the separation of the following stages

B—Pyknosis The cell is shrunken, angular in shape, so darkly staining as to obscure the condition of the nucleus and cell contents

A—Hyperchromatism The cell is round, the Nissl particles larger, more heavily staining than normal This stage is illustrated in Fig 1a

N—Normal The cell is round The Nissl particles may vary widely in size from cell to cell but are always discrete and are uniform

in size within any specific cell Fig 1b

1—Mild chromatolysis The Nissl particles are dustlike in some parts of the cell but the normal pattern may still be observed in the perinuclear region Fig 1c

2—Severe chromatolysis The Nissl particles are dustlike throughout the cell In some cases the nucleus is displaced to a peripheral position and only a dark margin of blue-staining material is visible Fig 1d

A series of polygons illustrating the conditions existing in each rabbit of the second series clarifies the nature of the response of trigeminal cells Fig 2 The reaction of these cells may be classified into 3 distinct phases of injury Initially, severe and extensive chromatolysis immediately follows irradiation 89-94% of the ganglion cells are completely chromatolyzed Not a normal cell may be found in the ganglion This condition was observed in all series As soon as exposure to irradiation is ended, however, a period of recovery sets in, so that as early as 3 hours after the end of the irradiation period, marked improvement can be noted, and by the end of 18 hours the ganglion cells have returned to an apparently normal condition At 43 hours the beginning of a second wave of chromatolysis is clearly observable The number of chromatolyzed cells and the severity of chromatolysis increases steadily until 82% of the large ganglion cells are affected at 13 days The second chromatolytic wave is sharply separable from that occurring during the irradiation period First, the onset

¹ Campbell B and Novick R, Proc Soc Exp Biol and Med 1946, 61, 425

² Campbell, B, Peterson, S C, and Novick, R Proc Soc Exp Biol and Med, 1946, 61, 353

CELL CHANGES IN TRIGEMINAL GANGLION

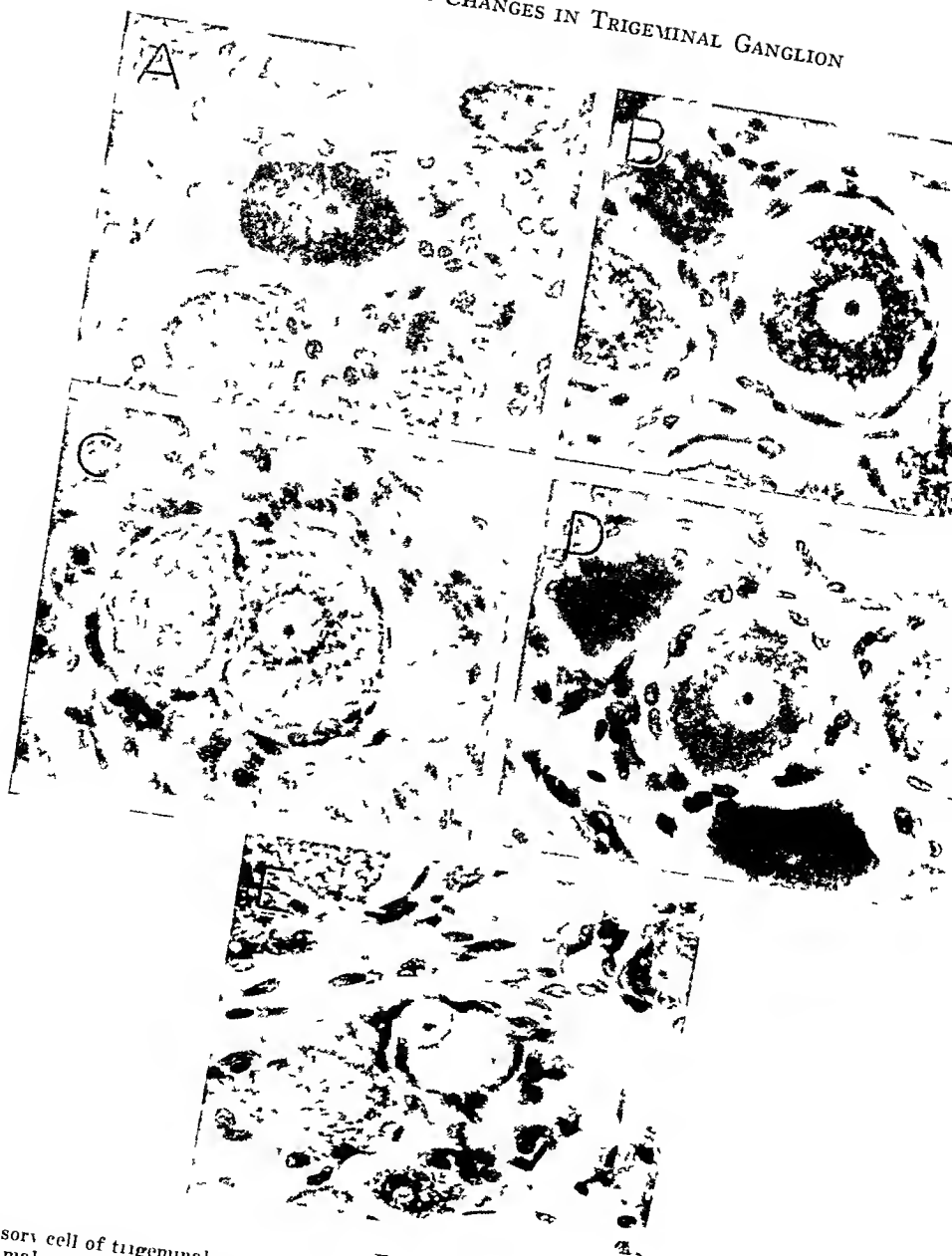


Fig 1

- a Sensory cell of trigeminal ganglion showing hyperchromatism
- b Normal sensory cell
- c Cell showing mild chromatolysis
- d Cell showing severe chromatolysis
- e Cell showing eccentric nucleus, severe chromatolysis $\times 400$

in order that a starting point in time for such correlation be determined. As the literature on the effects of irradiation on the nervous system has not included experiments

incorporating these conditions, a study applying the quantitative method of Campbell and Novick¹ was made of the sensory cells of the trigeminal ganglion of the rabbits re-

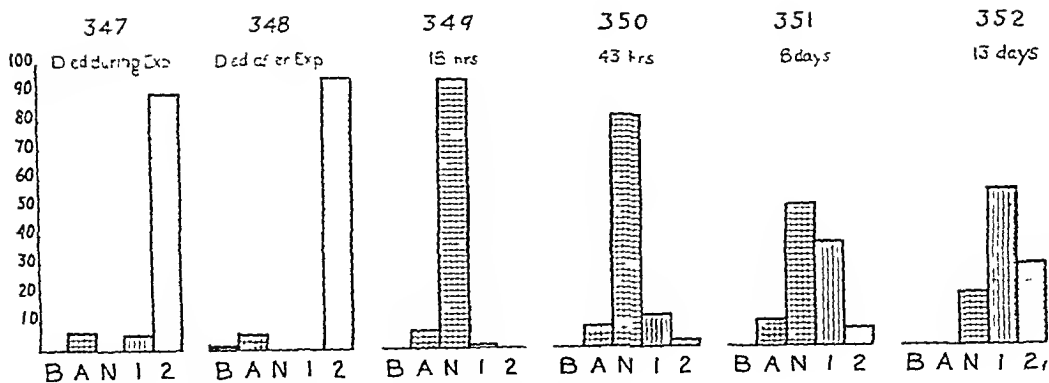


Fig 2

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A series of polygons illustrating the conditions existing in each rabbit of the second series clarifies the nature of the response of trigeminal cells Fig 2 The reaction of these cells may be classified into 3 distinct phases of injury Initially, severe and extensive chromatolysis immediately follows irradiation 89-94% of the ganglion cells are completely chromatolyzed Not a normal cell may be found in the ganglion This condition was observed in all series As soon as exposure to irradiation is ended, however, a period of recovery sets in, so that as early as 3 hours after the end of the irradiation period, marked improvement can be noted, and by the end of 18 hours the ganglion cells have returned to an apparently normal condition At 43 hours the beginning of a second wave of chromatolysis is clearly observable The number of chromatolyzed cells and the severity of chromatolysis increases steadily until 82% of the large ganglion cells are affected at 13 days The second chromatolytic wave is sharply separable from that occurring during the irradiation period First, the onset

¹ Campbell B and Novick R, *Proc Soc Exp Biol AND Med* 1946, 61, 425

² Campbell B, Peterson, S C, and Novick, R, *Proc Soc Exp Biol AND Med* 1946, 61, 353

is delayed until 43 hours after irradiation, and is preceded by a normal condition. Second, the onset is not simultaneous in each cell of the ganglion. The chromatolysis having once begun, progresses slowly. Initially it usually appears at a point halfway between the nucleus and the cell membrane and gradually spreads both centrally and peripherally, eventually involving the entire cell body. Some cells are so severely affected that their nuclei are displaced to a peripheral position and the Nissl substance entirely disappears, leaving only a blue-staining border, Fig 1e. Neuronophagia of dead ganglion cells may be observed in animals surviving for as long as 13 days. The second wave of chromatolysis is sufficiently persistent as to be discernible even after as long as 69 days.

The parallelism between the condition of the trigeminal ganglion cells and the clinical picture presented by the rabbits is marked. At the end of the exposure the cells were severely damaged. At this time the rabbits appeared acutely ill, displaying dyspnea, exophthalmos, somnolence and postural abnormalities. They improved very rapidly, however, and after 12 hours seemed almost normal. Simultaneously, the cells underwent

rapid recovery. The second chromatolytic wave was paralleled by a second phase of illness in the animals. They became emaciated and salivated excessively.

Discussion. In an investigation of radiation effects on the dorsal root ganglia of rats,³ Ma and Hsu concluded that the effects of Roentgen rays were indirect only, and that the pathological condition of these cells was caused by a disturbance in the nutrition of the cells as a result of vascular injury. The rapid onset of severe pathology in the trigeminal cells following irradiation indicates a direct relationship. This does not exclude the possibility, however, that injury of the cell is resultant upon injury of its axon rather than of the cell body itself.

Conclusions. Exposure of trigeminal ganglion cells to roentgen rays has been found to produce injury which may be separable into 3 phases:

1. Rapid phase of acute chromatolysis immediately following irradiation.
2. Recovery period.
3. Phase of the widely variable, slowly progressing spectrum of chronic chromatolysis.

³ Ma, W. C., and Chen Liang Hsu, *Am J Cancer*, 1940, **40**, 335.

15321

Bone Growth in Paralyzed Limbs *

W. D. ARMSTRONG

From the Division of Physiological Chemistry, University of Minnesota, Minneapolis, Minn.

Paralysis of an extremity beginning at an early age frequently results in disturbances of bone growth. A bone of the affected extremity may be shorter than that of the normal limb and, as demonstrated by the roentgenogram, its diameter and the thickness of its cortex and trabeculae may be reduced. Pottorff¹ and Howell,² each of whom used

one young dog as an experimental subject, investigated the changes occurring in the dimensions and structure of the bones of the forelimb after section of unspecified nerves of the brachial plexus. Howell's animal was allowed to live 19 weeks after paralysis of the right forelimb was produced and he found that the humerus and ulna of that limb were approximately 5% shorter than those of the normal limb. However, the diameter of the humerus of the paralyzed limb was much less than that of the normal limb and ap-

* This work was supported by a grant from the Josiah Macy, Jr., Foundation.

¹ Pottorff, J. L., *Anat Rec*, 1916, **10**, 234.

² Howell, J. A., *Anat Rec*, 1917, **13**, 233.

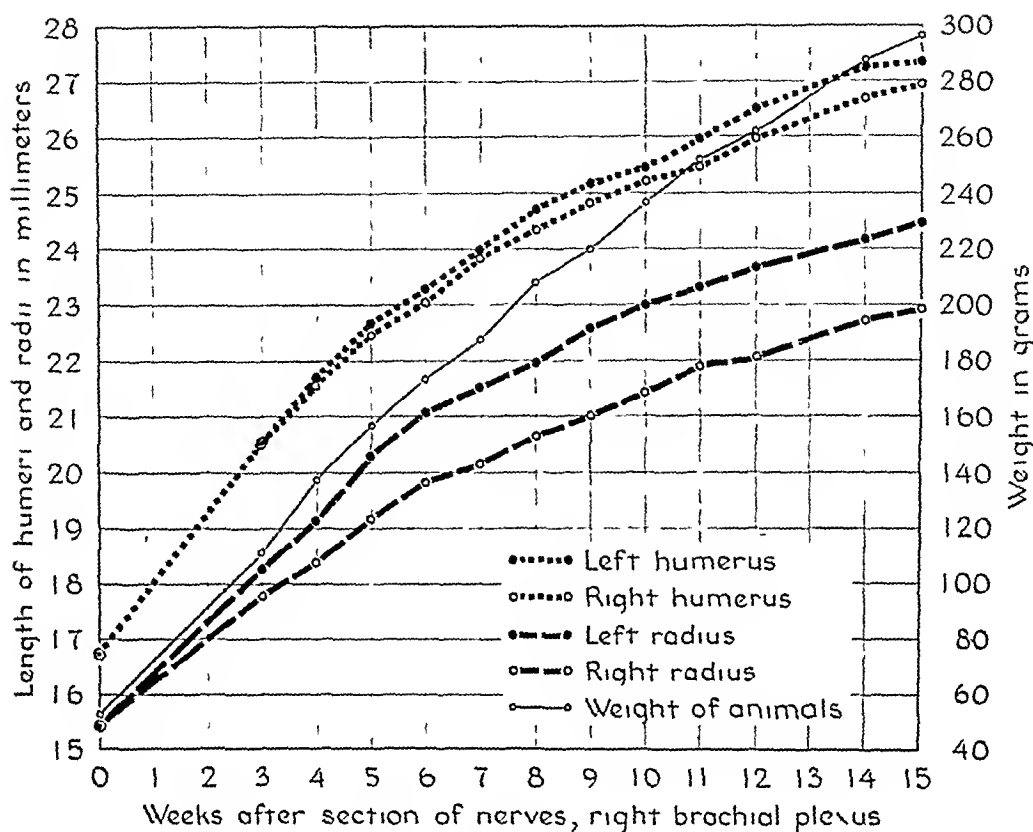


Fig 1

Bone growth in normal and paralyzed limbs of the rat

peared to have suffered an actual decrease in diameter. Howell interpreted his observations to indicate that mechanical factors of stress and strain have a pronounced effect on the growth of bone, but are not the sole factors in the regulation of bone growth.

Riley, McCleary and Johnson³ found the uptake of radioactive phosphorus by the bone ash of the humeri of normal and paralyzed limbs of rats, weighing between 150-200 g, to be equal and produced evidence to indicate that the humeri of the paralyzed limbs continued to gain in mass but at a rate slower than the normal. Allison and Brooks,⁴ using roentgenographic and histologic techniques, noted the gross and minute changes in

the atrophic bones of the forelimbs of dogs in which paralysis was produced by section of nerves after the animals were mature. Armstrong, Knowlton and Gouze⁵ determined the degree of atrophy which occurred in the humeri of mature rats following 21 days of brachial plexus paralysis and investigated the effect of certain steroid hormones on the composition and amount of bone in the humeri of the normal and paralyzed limbs.

In the present study, the length of the bones of the forelimbs was determined at intervals of time in rats in which paralysis of one arm was produced at an early age and the animals allowed to live to maturity. The initial and final diameters of the humeri were measured and data were collected to

³ Riley, F. R., McCleary, B., and Johnson, R. E., *Am. J. Physiol.*, 1945, **143**, 677.

⁴ Allison, H., and Brooks, B., *Surg., Gyn., and Obst.* 1921, **33**, 250.

⁵ Armstrong, W. D., Knowlton, J., and Gouze, M., *Endocrinology*, 1945, **36**, 313.

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¹ Pottorf, J. L., *Anat Rec*, 1916, 10, 234.

² Howell, J. A., *Anat Rec*, 1917, 13, 233.

TABLE I
Effects of Paralysis on Bones of the Forelimb of Rats *

	Humeri		Radii		Ulnae	
	Normal limbs	Paralyzed limbs	Normal limbs	Paralyzed limbs	Normal limbs	Paralyzed limbs
Length (mm)	27.57 ±0.231	26.91 ±0.249	24.47 ±0.148	22.84 ±0.190		
Diameter of shaft (mm)	2.85† ±0.039	2.52† ±0.005				
Diameter of head (mm)	5.59‡ ±0.055	5.39‡ ±0.057				
Ash content (%)	68.69 ±0.158	68.33 ±0.147	70.63 ±0.188	69.28 ±0.343	70.80 ±0.244	69.94 ±0.287
Dry, fat free weight (mg)	215.2 ±3.09	165.9 ±4.71	48.8 ±1.24	42.8 ±1.31	94.1 ±1.77	65.0 ±1.99
Difference (%)		22.9		27.2		30.9
Ash weight (mg)	147.8 ±2.40	113.4 ±3.30	41.5 ±0.64	29.7 ±0.77	66.6 ±1.05	45.5 ±1.37
Difference (%)		23.3		28.4		31.7

* The data are reported as means ± the standard error

† Initial diameter of shaft 1.91 ± 0.037 mm

‡ Initial diameter of head 4.92 ± 0.042 mm

same limbs. This finding may be due to the possibility that a more complete denervation of the muscles moving the forearm was obtained than of those having an action directly, or indirectly through the scapula, on the upper arm. In this connection, it is to be noted in Table I that the forearm bones of the paralyzed limbs exhibited a greater difference of dry, fat-free weight and of ash weight from the corresponding normal bones than was the case with the humeri.

It will be seen that while the mean final length of the humeri of the paralyzed limbs was slightly (1.7%) less than that of the normal limbs, the diameters of the shaft⁶ and head of these bones were very much less than those of the normal humeri. Although appositional growth was reduced more than epiphyseal growth, the first process was not prevented as is seen by a comparison of the initial and final diameters of the shaft and head of the humeri. The consistently lower ash content of the bones of the paralyzed limbs is in agreement with the results of experiments in which the paralysis of one

arm was produced in mature animals.⁷

It will be noted in Fig. 1 that the rate of growth of the humeri of both limbs was very greatly reduced at the end of the experiment when the animals were 132 days old. This finding indicates that these bones had reached or very nearly reached, their maximum length, a conclusion which is strengthened by the fact that the final roentgenograms showed that the heads of the humeri had practically complete bony union with the shafts. The curves showing the rate of growth of the radii, however, indicate that the bones were still growing at the end of the experiment. As mentioned above, the distal epiphyses of the radii and ulnae were found to lack bony union to the shaft when the animals were sacrificed. These findings indicate that skeletal growth in the rat has not ceased in animals with a body weight which is usually considered to be that of a mature animal.

Summary. Fifteen weeks after the forelimbs of young rats were denervated, it was found (1) The growth in length of the radii was affected earlier and to a greater degree than that of the humerus. (2) The reduction of growth in diameter of the humeri was greater than the reduction of growth in length. (3) Growth in diameter of the bones was reduced but not prevented. (4) The

⁶ The directly determined diameters of the mid point of the humeri measured in a plane perpendicular to that of the deltoid tubercle and at a right angle to the dimension measured from the films were: Humeri of normal limbs, 2.20 ± 0.038 mm and humeri of paralyzed limbs, 1.75 ± 0.071 mm.

demonstrate the differences in amount of bone and of bone ash in the skeleton of the normal limbs

Experimental Eight male rats of the Sprague-Dawley strain, when 27 days of age and with an average weight of 53.9 ± 0.91 g, were subjected to an operation in which certain nerves were severed in order to produce paralysis of the right forelimb. The animals were anesthetized by the intraperitoneal injection of 50 mg sodium pentobarbital per kilo of body weight and, using aseptic technique, the following nerves of the right brachial plexus were severed at the axillary level: median, ulnar, radial, axillary, musculocutaneous, subscapular, thoracodorsal and suprascapular.⁶ The animals were fed *ad libitum* for 15 weeks on a diet of 60% sucrose, 16.9% hydrogenated vegetable fat (Crisco), 18% lactalbumin (Borden's No 15-42), 4% salt mixture (Wesson's), 1% liver extract (Wilson's Fraction D) and 0.1% choline hydrochloride. In addition, certain crystalline compounds of the vitamin B group were incorporated in the diet[†] and the animals were given twice weekly 2 drops of a concentrate of vitamins A and D. The incisor teeth were kept cut to about half normal length to prevent the animals from chewing the paralyzed extremity.

At intervals of time, beginning with the day of operation, as indicated in Fig 1, the animals were anesthetized by the intraperitoneal injection of sodium pentobarbital and roentgenograms of both forelimbs were prepared. The extended limb, with the animal lying on its side, was held flat against the cardboard film holder and a target-to-film distance of 65 cm was used. The greatest length of the shadows of the humeri and of the radii were measured on the developed films, using a pair of finely pointed dividers and an accurately engraved ruler.[‡]

These measurements were recorded to the nearest 0.25 mm. The diameters of the shadows of the humeri at the midpoint of the shaft (in the plane of the deltoid tubercle) and of the proximal epiphysis (head) of the same bones were measured on the initial and final films. These latter measurements were made with a vernier caliper which was read to the nearest 0.1 mm. All measurements of length or diameter were made by 2 observers working independently. When the results obtained by the 2 observers pertaining to a particular dimension were not identical, the measurements were repeated until agreement was reached.

The animals were sacrificed 15 weeks after the operation. The forelimbs were removed and the bones of the arms and forearms were cleaned of soft tissue after prolonged boiling in distilled water. The distal epiphyses of the radii and ulnae, which were small, were ununited to the shaft and were lost in cleaning the bones. The bones were dried and extracted for 48 hours with an equal part mixture of alcohol and ether in a Soxhlet apparatus. After the bones were redried and weighed, they were ashed in platinum dishes at 700° to constant weight.

Results and Discussion The mean weight of the animals when sacrificed was 296.1 ± 10.2 g. Fig 1 summarizes the results as to the rate of increase of body weight and of growth in length of the humeri and radii. The data referring to dimensions of the bones given in Table I are the mean results of measurements made from the final roentgenograms. As seen from Fig 1, the growth in length of the radii of the paralyzed limbs was affected earlier and to a greater degree than was the case with the humeri of the

grms made on the day the animals were sacrificed showed that the shadows were, on an average, 1.2% longer than the true length of the bones. This exaggeration of the length of bone due to divergence of the X-ray beam probably represents the largest error, due to this factor, among the measurements made from the X-ray films. The effect of divergence of the X-ray beam on the length of the shadows of the bones would be reduced in the case of the films made when the bones were shorter.

⁶ Greene, E. C., *Trans Am Physiol Soc, N S*, 1935, 27, 1.

[†] Per 100 g of diet: 200 µg thiamine hydrochloride, 200 µg riboflavin, 200 µg pyridoxine hydrochloride, 400 µg calcium pantothenate.

[‡] Comparison of the directly measured length of the humeri of the normal limbs with the length of the shadows of these bones on the roentgeno-

TABLE I
Serial Inoculations in Mice in Preenecrotic Period C3H Tumor to ZBC Mice Without Milk Factor

Serial passage	Interval between inoculations (days)	Mice injected	Mice held	No of tumors	Time of appearance of 1st tumor (mo)	Progeny produced	No of tumors	Time of appearance of 1st tumor (mo)
Original	—	30	15	15	10	34	29	82
1st	72	30	15	11	7.5	30	29	80
2nd	53	30	15	6	7.5	24	0	—*
3rd	90	30	15	0	—*	26	0	—*
4th	71	30	15	0	—*	19	0	—*

* Period of observation 7 to 12 months

0.25 g. The appearance of tumors in the inoculated mice and in the first-litter progeny of those used for transmission material is shown with other pertinent data in Table I.

In the first group of mice all 15 females, which received the original mammary tumor filtrate developed tumors, 29 of the 34 progeny that did not receive the injection also developed tumors. The appearance of tumors in these 2 groups in 10 months and 82 months, respectively demonstrates the high susceptibility of the hybrid ZBC mice to the virus as isolated from the tumor. The number of tumors in the inoculated female mice dropped to 11 after the first transfer from lactating mammary glands, and to 6 in the second transfer, with no tumors appearing in later generations. It seems of some significance that although few tumors developed in the first and second generations, the time of their appearance was considerably shorter than in the original group and shorter than might be expected if the tumors had been spontaneous ones appearing in mice that nursed naturally infected females. This fact,

together with the appearance of early developing tumors and then a sudden disappearance of tumor formation in the progeny of the second generation, makes it seem that a relationship may exist between the earlier development and the decline in the number of tumors.

The procedure followed in these experiments seems to differ from natural transmission principally in the manner of introduction of the milk agent, in the delay of a few weeks in transfer, and possibly in the dosage, both as to size and repetition. It is not clear at this time whether the disappearance of tumor formation is related to the fact that a hybrid strain of mice was used, to a loss or a modification of the virus itself, or to a change in immunologic relationships.

Summary In serial passage of the milk agent, or virus, through highly susceptible ZBC hybrid mice by the intraperitoneal injection of mammary gland filtrates, the number of tumors declined in the first and second passages, and no tumors developed in the third and fourth passages.

quantity of bone and of bone salt was reduced by about 25% (5) The humeri, but not the radii, of both the normal and paralyzed limbs of rats 132 days of age, had practically ceased to grow in length

It is concluded that paralysis of a limb interferes more with appositional growth of bones than with epiphyseal growth and that bone growth is not prevented in a denervated limb

15322 P

Serial Transmission of the Milk Agent of Mouse Mammary Carcinoma*

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In a colony of inbred mice bearing breast tumors, the milk agent,¹ or virus,² is transmitted from mother to daughter by nursing. Mammary tumors indicating the presence of the milk agent do not appear in the mother until after the milk agent has been transmitted to the young. Thus, the milk agent in perpetuation is passed serially by nursing during the precancerous stage.

We have attempted, by artificial injection, to pass the milk agent of mouse mammary carcinoma through a series of susceptible mice in a manner similar to the usual mode of transmission in the precancerous stage. There was a decline of the tumor formation in the second passage and entire disappearance of tumors in the third passage. The mice inoculated in series were of the ZBC strain, a hybrid susceptible to the milk agent but not infected with it.

The serial inoculation was begun with a filtrate of spontaneous tumor from C3H mice. For the serial transmission of the agent, or virus, through the ZBC mice, filtrates of lactating mammary tissue were used as the inoculant. The general procedure was to

inoculate 30 mice in each generation, 15 of which we then held indefinitely and force-bred in order to induce maximum production of cancer. After allowing the other 15 inoculated mice to raise one litter, we killed them to obtain lactating mammary tissue to be used for inoculation of the next 30 mice in series. Females of the litters so produced were also force-bred for observations on cancer production.

In preparing the inoculant for the original group of 30 ZBC mice, 15 g of spontaneous tumor from C3H mice was ground with a mortar and pestle and suspended in 25 cc of 0.9% saline solution. The suspension was centrifuged 10 minutes at 2000 r p m so that the coarsely ground residue was removed. The supernatant fluid was then filtered through a tested Berkefeld filter, and the filtrate was injected into ZBC mice one month of age. Each mouse received by intraperitoneal inoculation a dose of 0.5 cc of filtrate, or the equivalent of 0.5 g of cancer tissue. For all subsequent inoculations, lactating glands obtained from female mice after birth of their first litter, were ground, centrifuged, filtered and injected in similar manner. The dosage, however, varied somewhat in each generation. In the first transfer generation in ZBC mice, the dose of filtrate for each animal was the equivalent of 0.25 g lactating mammary tissue. In the second generation, the dose of filtrate was equivalent to 0.33 g of mammary tissue and in the third and subsequent generations, equivalent to

* This investigation has been aided by grants from the Jane Coffin Childs Memorial Fund for Medical Research, the Cancer Research Fund of the Graduate School of the University of Minnesota, and the Citizens Aid Society of Minneapolis.

¹ Bittner, J. J., *Science*, 1936, **84**, 162.

² Green, R. G., Moosey, M. M., and Bittner, J. J., *Proc. Soc. Exp. Biol. and Med.*, 1946, **61**, 115.

TABLE I
Nicotinamide Output in mg/24 Hours Without Metabolism of Following Oral Administration of Nicotinamide, Nicotinic Acid or Cotinine

Medication given	A Full term newborn infants									
	None			100 mg			100 mg			Total for 3 days mg
	No of cases	Avg age in hrs	Avg daily output mg	No of cases	Avg age in hrs	Output 1st day mg	No of cases	Output 2nd day mg	No of cases	
None	2†	13	3.2 (0.7-7.1)							
N Amide				15	16	31.2 (12.1-72.1)	15	51.7 (10.3-93.0)	15	25.8 (2.9-54.7) 108.7 (28.9-178.5)
N Acid				11	14	11.7 (3.0-28.5)	11	29.8 (2.5-57.5)	11	20.8 (7.5-42.0) 65.3 (20.0-120.8)
Cotinine*				11	20	4.9 (1.3-8.8)	11	6.8 (1.3-9.6)	11	12.0 (5.2-22.7) 23.7 (7.8-37.2)
	B Premature infants									
	None			100 mg			100 mg			
	No of cases	Avg age in hrs	Avg daily output mg	No of cases	Avg age in hrs	Output 1st day mg	No of cases	Output 2nd day mg	No of cases	
N Amide	7†	11	1.7 (0.4-4.4)	7	12	20.5 (1.5-87.0)	7	37.8 (3.8-131.0)	7	11.5 (2.6-27.0) 71.9 (10.6-236.6)
N Amide	15†	11	1.7 (0.5-6)	13	12	22.5 (1.7-87.0)	11	36.5 (3.8-133.0)		

* Cotinine was administered in doses equivalent to 100 mg nicotinamide on the basis of molecular weight

† This series represents the data from 7 premature infants from whom we were able to obtain 24 hour urine samples for 4 consecutive days

‡ This series includes the 7 above cases and several additional cases from whom we were unable to obtain 4 consecutive 24 hour urine samples

Metabolism of Nicotinamide, Nicotinic Acid and Diethylamide of Nicotinic Acid (Coramine) by Newborn and Premature Infants

R A COULSON AND C A STEWART (Introduced by F G Brazda)

From the Departments of Biochemistry and Pediatrics, Louisiana State University School of Medicine, and The Charity Hospital of Louisiana, New Orleans

Najjar and Wood¹ reported the discovery of a compound in normal urine which is rendered fluorescent by the addition of alkali and butanol. Subsequent investigators^{2,3} have indicated that this substance is nicotinamide methochloride (N¹ methyl nicotinamide) and have demonstrated that its excretion is augmented by the ingestion of nicotinamide, nicotinic acid and coramine. Ellinger and Coulson⁴ studied the relative rate of elimination of nicotinamide methochloride following administration of these compounds to normal adults and concluded that nicotinamide is directly methylated whereas coramine and nicotinic acid are first converted to nicotinamide and are then methylated.

Since practically nothing is known relative to the metabolism of nicotinic acid and its derivatives by newborn and premature infants the present study was undertaken. The living conditions, diet, etc., of the young patients are well standardized and consequently this study was conducted under experimental conditions which were distinctly more uniform than those which prevailed in the investigations which have been made on adults.

Experimental Studies on Normal Newborn Infants. Colored newborn male infants were selected for the first experiment. These babies were considered full term on the basis of their general appearance and their birth weights, which averaged 7.1 lb (5.2-9.8 lb).

The infants were maintained under the ordinary conditions which prevail in a hospital ward where aseptic nursing is practiced. The babies received sterile water the first day of life and from 4 to 8 ounces of a sterile formula consisting of $\frac{1}{3}$ evaporated milk, $\frac{2}{3}$ water and 5% karo corn syrup the second day. Subsequently the food intake gradually increased to a maximum of 24 ounces of the formula per 24 hours by the eighth day. Twenty-four-hour urine specimens were obtained* by fastening a rubber tube over the penis with adhesive tape and collecting the urine in a loosely stoppered bottle which contained 5 ml of glacial acetic acid as a preservative. At the close of each 24-hour period the lower abdomen was depressed manually to force as much of the residual urine as possible from the bladder. Nicotinamide, nicotinic acid or coramine was given by mouth at 8 a.m. to some of the infants whereas other babies who received no medication served as controls. The urinary methochloride was determined by the method of Coulson, *et al.*⁵ which involves the adsorption of the methochloride on Decalco, elution with KCl and the subsequent development of fluorescence by alkaline isobutanol.

After experimenting with a number of nicotinamide "saturation" tests in adults Ellinger and Benesch⁶ adopted the total 24-hour output of nicotinamide methochloride following a single oral test dose of 100 mg of nicotinamide as perhaps the most reliable index of the nutritional level with respect to

¹ Najjar, V. A., and Wood, R. W., *Proc. Soc. Exp. Biol. and Med.*, 1940, **44**, 386.

² Huff, J. W., and Perlzweig, W. A., *Science*, 1943, **97**, 538.

³ Ellinger, P., and Coulson, R. A., *Nature*, 1943, **152**, 383.

⁴ Ellinger, P., and Coulson, R. A., *Biochem. J.*, 1944, **38**, 265.

* We are indebted to Miss Hazel Pierce and Miss Tallulah Moore for aid in the collection of urine samples.

⁵ Coulson, R. A., Ellinger, P., and Holden, M., *Biochem. J.*, 1944, **38**, 150.

⁶ Ellinger, P., and Benesch, R., *Lancet*, 1945, **2**, 197.

data from the remaining 8 infants included in the study are incomplete. On the first day of the experiment the average age and weight of these infants was 11 days and 4.2 (3.2-5.0) lb., respectively. The quantity of food these infants received varied according to age and body weight but in only one instance did the cow's milk consumption exceed 250 cc in 24 hours. It is apparent that the diet furnished them with less than a milligram of nicotinamide a day.

The results of the estimation of the methochloride elimination by the premature infants appear in Table I B. The average value for the first day of the study (11th day of life) was distinctly lower than that for the 23 newborn full term control infants. Throughout the period of study the increase in the excretion of nicotinamide methochloride following the administration of nicotinamide to the premature infants also averaged consistently below that observed in the full term infants given equal doses of the compound. Although one premature eliminated 236.6 mg of the methochloride after a total intake of 200 mg of nicotinamide this was the only case where the excretion over the 3-day period exceeded 71.9 mg the average total excretion for the entire group (Table I B). In spite of the fact that as compared with premature infants the absolute excretion was much greater in the case of the normal newborn infants the relative per cent of the 3-day total excreted each day was nearly the same in both normal infants and prematures. No correlation was found between the total methochloride elimination and the daily urine volumes. The average urine volume of the prematures was 85.4 ml/24 hours and that of the newborn full term infants 91.6 ml on the days of the experiment.

Effect of Methionine on Nicotinamide Methochloride Elimination The role of methionine in methylation has been studied by Perlzweig *et al.*⁸ who have presented evidence that this substance is involved in the methylation of nicotinamide. We recognized the possibility that the low values for nico-

TABLE II
The 24 Hour Nicotinamide Methochloride Output of Newborn Infants Following One Dose of 100 mg of Nicotinamide and 0.6 g Methionine

Case	24 hr urine vol	Nicotinamide methochloride output in mg/24 hr
M B	125	37.5
E R	205	28.7
O S	84	65.5
M T	118	48.4
L D	156	27.3
L T	87	15.2
M C	90	24.8
H F	38	11.4
	avg 112.9 ml	avg 32.4 mg

tinamide methochloride excretion obtained for some of our infants might be due to a deficiency in their body store of methionine rather than in the inherited store of nicotinamide. Consequently 8 infants were given 0.3 g each of methionine 20 hours before the experiment started and an additional 0.3 g at the time the 100 mg dose of nicotinamide was administered. The results which appear in Table II show an average 24-hour excretion of 32.4 mg of nicotinamide methochloride. Since this value does not differ significantly from that observed for the 15 infants who received 100 mg of nicotinamide but no methionine (Table I A) it appears that in the quantity given methionine had no appreciable effect on the excretion of nicotinamide methochloride. It is possible that larger doses of methionine administered over a longer period might yield different results.

The Metabolism of Coramine The slow and relatively incomplete conversion of coramine to what is apparently nicotinamide methochloride leads us to suspect that coramine might be eliminated, at least in part, as coramine methochloride. To test this hypothesis coramine methochloride was synthesized by the methylation of coramine in methanol by methyl iodide, and the subsequent conversion of the iodide to the chloride by the action of silver chloride. Coramine methochloride proved to be non-fluorescent in the filtered 366 mμ line from a quartz mercury vapor lamp. The addition of NaOH and isobutanol converts coramine methochloride into a fluorescent derivative, the hue of which is indistinguishable from that

⁸ Perlzweig, W. A., Bernheim, M. L. C., and Bernheim, F., *J. Biol. Chem.*, 1943, **150**, 401.

this item. The present authors applied this test to 24 full term newborn infants for the first or preliminary experiment. These infants whose records do not appear in the tables eliminated an average of 36.7 mg (11.5-95.0 mg) of the methochloride in the 24-hour period which immediately followed the oral administration of the single test dose. This excretion was more than 10 times that eliminated per 24 hours by control infants (Table I A) who did not receive nicotinamide and who served as normal controls for later studies.

It was our opinion that the relative degree of "saturation" might better be determined by analysis of the 24-hour urine samples following 2 successive doses given 24 hours apart. Consequently additional experiments were conducted on infants who received either 100 mg of nicotinamide or nicotinic acid or doses of coramine which on the basis of its molecular weight were equivalent to 100 mg of nicotinamide. These compounds were administered on each of the first 2 days of life. On the following day, during which no medication was administered, a third urine sample was collected and its methochloride content was also determined. The daily urinary titers of nicotinamide methochloride excreted by these cases as well as by the controls who received no medication appear in Table I A.

The total nicotinamide content of the small quantity of formula the infants consumed probably was negligible if we are to accept the values Cheldelin and Williams⁷ obtained for the nicotinamide content of cow's milk. Apparently the intestinal flora or the tissue stores were the chief sources of nicotinamide for these infants until it was administered orally.

The 23 control infants eliminated an average of 3.2 mg of the methochloride during the first day of life (Table I A). This average is distinctly lower than those observed for the first 24-hour period following the administration of nicotinamide, nicotinic acid or coramine. The data in Table I A indicate that nicotinamide has a greater im-

mediate influence on elevating the methochloride output than nicotinic acid and that nicotinic acid is considerably more effective in this respect than coramine. These observations for infants are in essential agreement with the findings of Coulson and Ellinger⁴ as to the relative influence of these compounds on the elimination of the methochloride in adults.

For each of the experimental groups the output of nicotinamide methochloride was significantly higher on the second day of medication than on the first day. On the third day during which no medication was given the urinary titer decreased considerably for the full term infants who had received nicotinamide or nicotinic acid but increased significantly for the group to which coramine was administered. Our results also show that with respect to their effect on the elimination of nicotinamide methochloride the relative positions of these 3 compounds remained unchanged throughout the 3-day period of observation covered by this study.

It would seem that like the adult, the newborn infants are able to methylate the preformed nicotinamide with relative ease and rapidly whereas they convert coramine slowly and somewhat less completely first to nicotinamide and then to nicotinamide methochloride. Nicotinic acid occupies an intermediate position in this regard. The absolute range of values for the methochloride elimination was very great in the nicotinamide and nicotinic acid groups but varied within more narrow limits in the infants who received coramine.

Premature Infants. Although special difficulties attend experimental studies on premature infants, nevertheless we obtained some information on their methochloride elimination before and following the administration of nicotinamide. In each instance an attempt was made to collect 4 consecutive 24-hour urine specimens, the first without the administration of nicotinamide, the second and third following 2 daily doses of 100 mg each and the fourth without medication. Complete observations were made in 7 cases (Table I B) but owing to vomiting and other disorders common to premature infants the

⁷ Cheldelin, V. H., and Williams, R. R., *Jour Nutrition*, 1943, 26, 417.

data from the remaining 8 infants included in the study are incomplete. On the first day of the experiment the average age and weight of these infants was 11 days and 4.2 (3.2-5.0) lb, respectively. The quantity of food these infants received varied according to age and body weight but in only one instance did the cow's milk consumption exceed 250 cc in 24 hours. It is apparent that the diet furnished them with less than a milligram of nicotinamide a day.

The results of the estimation of the methochloride elimination by the premature infants appear in Table I B. The average value for the first day of the study (11th day of life) was distinctly lower than that for the 23 newborn full term control infants. Throughout the period of study the increase in the excretion of nicotinamide methochloride following the administration of nicotinamide to the premature infants also averaged consistently below that observed in the full term infants given equal doses of the compound. Although one premature eliminated 236.6 mg of the methochloride after a total intake of 200 mg of nicotinamide this was the only case where the excretion over the 3-day period exceeded 71.9 mg; the average total excretion for the entire group (Table I B). In spite of the fact that as compared with premature infants the absolute excretion was much greater in the case of the normal newborn infants the relative per cent of the 3-day total excreted each day was nearly the same in both normal infants and prematures. No correlation was found between the total methochloride elimination and the daily urine volumes. The average urine volume of the prematures was 85.4 ml/24 hours and that of the newborn full term infants 91.6 ml on the days of the experiment.

Effect of Methionine on Nicotinamide Methochloride Elimination The role of methionine in methylation has been studied by Perlzweig *et al*⁸ who have presented evidence that this substance is involved in the methylation of nicotinamide. We recognized the possibility that the low values for nico-

TABLE II
The 24 Hour Nicotinamide Methochloride Output of Newborn Infants Following One Dose of 100 mg of Nicotinamide and 0.6 g Methionine

Case	24 hr urine vol	Nicotinamide methochloride output in mg/24 hr
M B	125	37.5
L R	205	28.7
O C	84	65.5
M T	118	48.4
L D	156	27.3
L T	87	15.2
M C	90	24.8
H F	38	11.4
Avg 112.9 ml		Avg 32.4 mg

tinamide methochloride excretion obtained for some of our infants might be due to a deficiency in their body store of methionine rather than in the inherited store of nicotinamide. Consequently 8 infants were given 0.3 g each of methionine 20 hours before the experiment started and an additional 0.3 g at the time the 100 mg dose of nicotinamide was administered. The results which appear in Table II show an average 24-hour excretion of 32.4 mg of nicotinamide methochloride. Since this value does not differ significantly from that observed for the 15 infants who received 100 mg of nicotinamide but no methionine (Table I A) it appears that in the quantity given methionine had no appreciable effect on the excretion of nicotinamide methochloride. It is possible that larger doses of methionine administered over a longer period might yield different results.

The Metabolism of Coramine The slow and relatively incomplete conversion of coramine to what is apparently nicotinamide methochloride leads us to suspect that coramine might be eliminated, at least in part, as coramine methochloride. To test this hypothesis coramine methochloride was synthesized by the methylation of coramine in methanol by methyl iodide, and the subsequent conversion of the iodide to the chloride by the action of silver chloride. Coramine methochloride proved to be non-fluorescent in the filtered 366 mμ line from a quartz mercury vapor lamp. The addition of NaOH and isobutanol converts coramine methochloride into a fluorescent derivative, the hue of which is indistinguishable from that

⁸ Perlzweig, W. A., Bernheim, M. L. C., and Bernheim, F., *J. Biol. Chem.*, 1943, 150, 401.

developed from nicotinamide methochloride by the same procedure. Coramine methochloride also resembles nicotinamide methochloride in that it is adsorbed on Decalso, eluted with KCl and as a consequence it may be estimated fluorometrically in the same manner as nicotinamide methochloride. Preliminary experiments have shown that the absolute intensity of the fluorescence of the derivative of coramine is, however, only about 12% of that developed from equivalent amounts of nicotinamide methochloride.

In order to test the effect of coramine methochloride on the fluorescence developed from the urine by the procedure of Coulson *et al.*⁹ for the estimation of nicotinamide methochloride an adult was given a single oral dose of 200 mg of coramine methochloride. No increase in fluorescence occurred. As a further check 2 normal newborn infants were each given 200 mg of coramine methochloride subcutaneously without increasing the total fluorescence developed from the urine by alkaline-isobutanol treatment. It would appear that if coramine is methylated in the human body and excreted as coramine methochloride this does not occur to any appreciable extent. It is more probable that coramine is normally converted to nicotinamide which in turn is methylated and excreted.

Discussion The nicotinamide produced by the intestinal flora is an important if not the chief source of this vitamin for the adult.^{9,10} Since, however, the average age of our newborn infants was less than 40 hours at the conclusion of the first day of the experiment, it is doubtful that the intestinal flora was sufficiently well established to provide them with any appreciable quantity of nicotinamide. Furthermore, since they received nothing but water for 24 hours and very small quantities of milk during the succeeding 10 hours they derived very little of the vitamin from the diet. Nevertheless, in spite of scant nicotinamide production in the

gut and a very low dietary intake the newborn infants studied excreted 3.2 mg nicotinamide methochloride in the urine, a quantity probably in excess of that derived from these 2 sources.

The nicotinamide which appears in the urine as the methochloride represents nicotinamide which was either synthesized by the infant or which diffused across the placenta to the fetus before parturition. The great variations in the daily methochloride excretions of newborn infants probably reflect either variations in the nutritional status of the mother or different degrees of permeability of the placental barrier. It is premature to suggest that those infants whose methochloride elimination was distinctly below the average found in this study are necessarily "deficient" in the pellagra-preventive compounds since very little is known of the absolute human adult nicotinamide requirements and nothing is known of the newborn. Since the methochloride content of newborn infants probably exceeds that which could have been attributed to nicotinamide derived from the diet or intestinal flora it is probable that the tissues of the newborn infant contain an appreciable store of nicotinamide at the time of birth.

The excretion of nicotinamide methochloride by the full term infants following the ingestion of nicotinamide, nicotinic acid or coramine is essentially equal to that observed by Ellinger and Coulson¹ for adults when given equal doses of the same compounds. This suggests that the absolute weight of normal liver tissue is not a factor in methylation. We can offer no explanation for the observation that under similar experimental conditions premature infants excrete less nicotinamide methochloride than full term infants.

Summary Experiments were conducted on newborn infants to determine the 24-hour elimination of nicotinamide methochloride under normal conditions or following the administration of nicotinamide, nicotinic acid or coramine. With regard to the influence of the 3 compounds tested on nicotinamide methochloride output nicotinamide proved to

⁹ Ellinger, P., Coulson, R. A., and Benesch, R., *Nature*, 1944, **154**, 270.

¹⁰ Ellinger, P., Benesch, R., and Kety, W. W., *Lancet*, 1945, **1**, 432.

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15324

Vitamin E Levels in Maternal and Fetal Blood Plasma

JON V. STRAUMFJORD AND MARY LOUISE QUARFEE (Introduced by Philip L. Harris)

From the Astoria Extension Research Laboratory (Department of Biochemistry, University of Oregon Medical School), Astoria, Oregon, and Research Department, Distillation Products, Inc., Rochester, N. Y.

Some insight into the role of vitamins in human physiology may be gained from a study of the concentration of vitamins in body tissues and fluids in various physiologic states. Among these are pregnancy, parturition and the neonatal state. The content of vitamin E in these states is of particular interest because of its well established function in insuring normal reproduction in certain laboratory animals.

Vitamin E is normally not present in the urine¹. Plasma, however, is readily available for investigation of vitamin E content.

The plasma levels of vitamin A in pregnant women and newborn infants have been reported in several papers.² These determinations became possible with the development of suitable clinical methods for determination of plasma vitamin A. A clinical method for the determination of plasma vitamin E³ has made possible a similar investigation of the maternal and fetal plasma levels.

The plasma levels of vitamin C, carotene,

vitamin A and vitamin E have been simultaneously determined in a fairly large series of normal persons and patients suffering from various disease processes. The levels found for vitamin A, carotene and vitamin C were in good general agreement with those previously reported. This report deals only with the levels found for vitamin E in the plasma of pregnant and parturient women and newborn infants.

Procedure and Methods. Plasma was obtained from oxalated blood from the cubital veins of women and from oxalated blood from the umbilical cord for infants' samples. The umbilical cord was clamped and divided a few minutes after delivery and the blood collected from the placental end of the cord before delivery of the placenta. The sample from the mother was obtained immediately after completion of the delivery—about one-half hour later than the cord sample. Vitamin E levels were determined by the method of Quarfee and Harris,³ using the hydrogenation technic of Quarfee and Biehler.⁴ A Coleman universal spectrophotometer model 11 was used. In brief, the method comprises extraction of plasma lipids with ethanol and petroleum ether, hydrogenation to obviate interference due to carotenoids and vitamin A, and application of the Emmerie and Engel color reaction to measure total tocopherols.

¹ Hines, L. R., and Mattill, H. A., *J. Biol. Chem.*, 1943, **149**, 549.

² Abt, Arthur F., and others, *Quart. Bull. Northwestern U. Med. School*, 1942, **16**, 245. Lund, C. J., and Kimble, M. S., *Am. J. Obstet. and Gynec.*, 1943, **46**, 207. Byrns, Jane N., and Eastman, N. J., *Bull. Johns Hopkins Hosp.*, 1943, **73**, 132. Bodinsky, Oscar, Lewis, J. M., and Lullienfeld, M. C. C., *J. Clin. Invest.*, 1943, **22**, 643.

³ Quarfee, M. L., and Harris, P. L., *J. Biol. Chem.*, 1944, **156**, 499.

⁴ Quarfee, M. L., and Biehler, R., *J. Biol. Chem.*, 1945, **159**, 663.

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(vitamin E) Pure natural α -tocopherol was used to prepare a standard curve, since β - and γ -tocopherols give substantially the same color response under the conditions used

Results with Statistical Analysis Values in Pregnant Women Plasma vitamin E values were determined on 23 women in various stages of pregnancy. Inspection of the results showed an increased level of tocopherols in the latter part of pregnancy. Eleven women in the first 24 weeks of pregnancy had a mean level of 1.17 mg% tocopherols with a standard deviation of 0.19. Twelve patients in the 25th to 36th week of pregnancy had a significantly higher mean level of 1.62 mg% with a standard deviation of 0.31. For men and non-pregnant women mean levels of plasma vitamin E of 1.05 mg% for a group in Rochester, New York, and 1.04 mg% for a group in Birmingham, Alabama, have been determined by the same method.⁵

Values in Mothers and the Newborn Plasma vitamin E was determined on 54 mothers and respective cord bloods at delivery. Inclusion of 2 sets of twins gave 56 values for infants. The mean maternal vitamin E was 1.70 with a standard deviation of 0.30. The mean fetal vitamin E was 0.34 with a standard deviation of 0.12.

Thus, vitamin E is similar to other fat-soluble vitamins, A and carotene, in showing markedly lower values in the infant than in the mother. The mean ratio of plasma vitamin E in the mother to that in the infant was 5.7. This figure is interesting in view of the conclusion of Mason and Bryan,⁶ based on bioassay of tissues, that for the rat "the concentration of vitamin E in the placenta and uterus of high-E mothers was about 5 times greater than in the full-term fetus or newborn."

The correlation coefficient of maternal and fetal vitamin E levels was calculated and showed absolutely no correlation between the 2.

⁵ Harris, P. L., Jensen, J. L., Hickman, K. C. D., and Spies, T., *Am J Pub Health*, 1946, **36**, 155.

⁶ Mason, K. E., and Bryan, W. L., *J Nutrition*, 1940, **20**, 501.

Relationships of Fetal Vitamin E The possibility of a sex difference in the plasma vitamin E levels of the newborn was investigated. Thirty female infants had a mean of 0.355 (S. D. = 0.145), and 26 males a mean of 0.318 (S. D. = 0.089). However, the difference is not statistically significant.

A lack of correlation between vitamin E in the infant and birth-weight was also observed.

Two sets of twins are included in the group. One pair, identical girls, showed vitamin E values of 0.42 and 0.48. A second pair, boy and girl, had identical levels of 0.30.

Comment These results differ considerably from the levels reported by Varangot.⁷ Varangot found that although the level in non-pregnant women was considerably lower than that in pregnant women, the level in the first trimester of pregnancy was higher than in the third. In 18 samples of blood collected from the umbilical vein at birth the vitamin E level was approximately 0.1 mg%. In 6 samples the amount was too small to measure. The serum vitamin E levels for non-pregnant women first reported by Varangot were much higher than those obtained with a later method in which he employed a chromatographic adsorption column to eliminate interfering substances. This method was used for the series referred to above. His results, however, agree with ours in respect to the much lower concentration of vitamin E in the fetal blood than in the maternal blood and the much higher concentration of vitamin E in the blood of pregnant than of non-pregnant women.

It has been shown that the blood lipids increase in pregnancy.⁸ Vitamin E accordingly behaves in a similar way. The concentration of lipids in the fetal plasma is generally much lower than in the maternal plasma⁹ which is likewise true of vitamin E.

⁷ Varangot, J., *Compt rend Acad d sc*, 1942, **211**, 691; Varangot, J., Chailley, H., and Rieux, N., *Compt rend Soc de biol*, 1943, **137**, 210; *Ibid*, *Compt rend Soc de biol*, 1943, **137**, 393.

⁸ Tyler, M., and Underhill, F. P., *J Biol Chem*, 1925, **60**, 1.

⁹ Slemons, J. M., and Stander, H. J., *Bull Johns Hopl ins Hosp*, 1923, **34**, 7; Boyd, Eldon M., and

This relationship has been shown to hold also for vitamin K as measured by prothrombin levels¹⁰

While this placental barrier may be regarded usually as a protective mechanism, the fact that neonatal hemorrhages may be prevented by the administration of additional vitamin K to mothers, whose plasma contains adequate amounts of vitamin K for their own needs, might indicate that the placental barrier does not always function in a beneficial way

Administration of supplements of vitamin E to the mother during pregnancy might possibly increase the fetal plasma vitamin E. Experiments to test this point are in progress.

"The umbilical vessels do offer a unique source of blood for analyses, but the results

Wilson, Karl M., *J. Clin. Investigation*, 1935, 14, 7

¹⁰ Smith H. P., and Warner, E. D., Vitamin K. Clinical Aspects, in Evans, E. A., Jr. *The Biological Action of the Vitamins*, University of Chicago Press, 1942, 211

obtained therefrom are a little too limited to an especially transient state of affairs to throw much light on the whole period.¹¹ The situation at delivery does not represent the relationship throughout gestation and broad metabolic and nutritional generalizations should not be drawn from determination of this kind although they are of interest in their own right and their significance becomes greater with advancing knowledge

Conclusions 1 The plasma vitamin E level tends to rise with the progress of pregnancy and is approximately 65% higher at term than that for non-pregnant women

2 The vitamin E level in the cord blood of infants at birth is much lower than the level in the venous blood of the mothers

3 No sex difference in vitamin E levels is apparent in newborn infants

¹¹ Smith, Clement A., *The Physiology of the Newborn Infant*, ed 1, Charles C Thomas 1945, 5

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Biosynthesis of Nicotinamide in Man*

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In previous studies^{1,2} we have observed that under certain conditions at least, synthesis of thiamine and of riboflavin occurred in the gastrointestinal tract of man. These studies were carried out on purified

diets in which the B factors were supplied almost entirely in the form of crystalline supplements. The recovery of these factors from the excreta was found to be in excess of the intake and in a number of experiments the excretion in the stools alone exceeded the quantity ingested. It was found that the administration of sulfasuxidine inhibited thiamine synthesis effectively, but caused no suppression of riboflavin synthesis, indeed, the synthesis of riboflavin appeared to be increased by the drug.

Experiments pointing to the synthesis of nicotinamide in the human intestine were

* The expenses of this study were defrayed in part by grants from the John and Mary R. Markle Foundation, the Sugar Research Foundation and from Merck Johnson & Co.

¹ Najjar, V. A., and Holt, L. E., Jr., *J. A. M. A.*, 1943, 123, 683

² Najjar, V. A., Johns, G. A., Medaury, G. C., Fleischmann, G., and Holt, L. E., Jr., *J. A. M. A.*, 1944, 126, 357

reported by Ellinger and his collaborators^{3,4} These authors did not employ purified diets but attempted to maintain a constant nicotinamide intake with natural foods containing nicotinamide Measurements were made of the excretion of N-methyl nicotinamide derivatives (F_2) in the urine, which were found to decrease markedly after the administration of sulfa drugs, notably sulfasuxidine, from this the authors deduced that a substantial amount of nicotinamide was being synthesized by the intestinal bacteria

It seemed to us desirable to study the biosynthesis of nicotinamide under conditions in which the intake was rigidly controlled and reduced to a minimum—namely, with purified diets It is the purpose of the present communication to report such studies We have been able to confirm the finding of Ellinger *et al* in regard to the biosynthesis of nicotinamide, but not in regard to the suppression of this phenomenon by sulfasuxidine

Experimental Subjects Studies were made on 4 adolescents between 10 and 14 years of age who were inmates of an institution The subjects lived a sedentary life during the period of study—approximately 3 months Temperature and pulse were recorded 4 times daily and physical examinations were done about once a week The subjects remained in excellent health throughout the study, they were examined with particular care for changes suggestive of pellagra, but no such changes were observed

Diet This consisted of vitamin-free casein,[†] crisco,[‡] dextrimaltose[§] and minerals mixed together to form a dough which was

fed in equal quantities at each of the 3 meals This diet provided approximately 40 calories per kilogram, distributed as follows protein 15%, fat 35% and carbohydrate 50% The mineral mixture was supplied in quantities of 1.5 g daily In order to insure accuracy of intake vitamin supplements were given independently Fat soluble vitamins were provided by a daily dose of 5 drops of cod liver oil concentrate (Mead Johnson) A mixture of water soluble vitamins[¶] containing no nicotinic acid was given in equal quantities 3 times a day Small amounts of nicotinic acid were found to be present in the basal diet, providing a maximum intake of 1.5 to 2.0 mg per day

Plan of Study The study was divided into 3 periods of approximately one month each During the first and third periods the nicotinic acid deficient diet was given alone, during the second period sulfasuxidine was added in doses of 1.5 g every 4 hours

Urine was collected throughout and 24-hour specimens were analyzed daily for N-methyl nicotinamide by the fluorometric method of Najar⁵

Results The output of N-methyl nicotinamide (F_2) in the urine for the 4 subjects is presented in Table I where daily average figures for each 6 days of the study are shown

It will be noted that the output of N-methyl nicotinamide on the experimental diet averaged close to 1 mg per day (extremes 0.16–2.25 mg), being somewhat less than values reported for active male adults on a

we are indebted to Dr Warren M. Cox, Jr, showed a content of 8.6 γ per g

¶ The mineral mixture used had the following composition (measured in g) NaCl 18.9, CaHPO_4 (anhydrous) 25.0, MgSO_4 (anhydrous) 6.86, KHCO_3 44.4, KCl 2.88, $\text{Fe}_3\text{Citrate}$ 2.21, CuSO_4 (anhydrous) 0.24, MnSO_4 (anhydrous) 0.15, KI 0.015 and NaF 0.03

¶ The mixture of water soluble vitamins provided the following daily quantities, ascorbic acid 25 mg, calcium pantothenate 1 mg, pyridoxine 1 mg, inositol 1 mg, p-aminobenzoic acid 1 mg, choline chloride 5 mg, thiamine 1 mg, and riboflavin 1 mg

⁵ Najar, V. A., *Bull. Johns Hopkins Hosp.*, 1944, 74, 392

³ Ellinger, P., Coulson, R. A., and Benesch, R., *Nature*, 1944, 154, 270

⁴ Ellinger, P., and Benesch, R., *Lancet*, 1945, 1, 432

[†] The vitamin free casein (Sheffield) used in the study contained 0.3 to 0.9 γ nicotine acid per g, according to assays made in the Sheffield laboratories

[‡] This was supplied through the courtesy of Procter and Gamble

[§] Dextrimaltose No. 2, used in this study was generously supplied by Mead Johnson and Co. Nicotinic acid assays of this product, for which

TABLE I
Urinary Output of N Methyl Nicotinamide Chloride (μg per Day)

The figures represent average daily excretions in successive 6 day periods							
		Subjects					
Diet		Date	Bu	Ge	Mi	Se	
Foreperiod on institutional diet		10 15 to 10 20 44	960	334	†	902	
Purified diet started		10 21					
Part I	<i>Nicotinamide deficient diet</i>	10 22 to 10 27	463	270	†	655	
		10 29 to 11 3	1065	246	†	1683	
		11 4 to 11 9	555	240	†	767	
		11 10 to 11 15	337	166	580	873	
		11 16 to 11 21	223	179	265	684	
Sulfasuxidine started		11 25					
Part II	<i>Nicotinamide deficient diet plus sulfasuxidine</i>	11 28 to 12 3	233	871	675	956	
		12 4 to 12 9	526	674	527	855	
		12 10 to 12 15	685	790	1404	1087	
		12 16 to 12 21	1914	2131	2250	1582	
		12 22 to 12 26*	1852	1642	1649	1792	
Sulfasuxidine stopped		12 26					
Part III	<i>Nicotinamide deficient diet</i>	12 27 to 1 1 '45	1599	874	1965	1582	
		1 2 to 1 7	649	408	1094	1105	
		1 8 to 1 13	509	†	852	1481	
		1 14 to 1 19	251	471	840	1362	

* This period represents an average of 5 days only

† Specimens lost during this period

normal diet (2 to 10 mg)^{6,7,8} and for control subjects on our purified diet (avg 4.28 mg) who received a daily supplement of 25 mg nicotinamide. However, the output on our experimental diet was comparable to that on the institutional diet, which was calculated to contain more than 10 times as much nicotinic acid. In other words a 10-fold reduction in nicotinic acid intake produced no appreciable reduction in the output of the methyl derivative in the urine.

Under conditions observed hitherto by various workers only a fraction of the ingested nicotinamide, varying from 10 to 40% appears in the urine as N-methyl derivative. In the present experiments, however, it is evident that at times the urinary excretion of N-methyl derivative is comparable to that of the ingested nicotinamide. This might be interpreted as indicating either (1) that

at the low level of intake in these experiments close to 100% of the ingested nicotinamide was excreted in the urine as the N-methyl product or (2) that a substantial synthesis of nicotinamide occurred within the body. We are inclined to dismiss the former possibility, because of the numerous observations now available that pellagrins on pellagra-producing diets and dogs on a black-tongue-producing diet show negligible quantities of N-methyl nicotinamide in the urine. Hence a reduction of nicotinamide intake *per se* is not followed by a decreased proportion of nicotinamide excretion in the form of N-methyl derivative and by the persistence of that derivative in the urine in considerable quantity, as occurred in the experiments here reported. The persistent excretion must therefore be attributed to biosynthesis of nicotinamide.

The effect of sulfasuxidine, which failed entirely to cause any reduction in N-methyl nicotinamide output, was somewhat of a surprise to us in view of the sharp reduction in output that had followed its use in the experiments of Ellinger *et al*. Indeed it ap-

⁶ Johnson, R. E., Henderson, C., Robinson, P. F., and Consolazio, F. C., *J. Nutr.* 1945, 30, 89.

⁷ Ellinger, P., and Benesch, R., *Lancet* 1945, 1, 197.

⁸ Michelsen, O., and Erickson, L. L., *Proc. Soc. Exp. Biol. and Med.*, 1945, 58, 33.

peared that in 3 of our 4 subjects there was an increased excretion of N-methyl derivative in the sulfasuxidine period with a tendency to decline when the drug was discontinued. The explanation of this discrepancy may well be in the nature of the basal diets, which permitted different bacterial flora to flourish when the coliform organisms were suppressed by the drug. In our experiments it may be assumed that the organisms which replaced the colon bacilli were capable of synthesizing nicotinamide whereas under the conditions of the British investigators organisms flourished which were incapable of performing this synthesis.

In a recent communication Mickelsen and Erickson⁸ reported some human experiments in which a considerable reduction in nicotinamide intake was followed by only a minimal reduction in the output of N-methyl derivative. This led them to question the value of F_2 excretion "as a means of evaluating the state of niacin nutrition." We believe that their data deserve a different interpretation in the light of our observations and those of Ellinger on the biosynthesis of nicotinamide. Under conditions in which biosynthesis occurs there is apparently little correlation between niacin intake and F_2 ex-

cretion, but we see in their data nothing to disprove the view that niacin nutrition is not reflected in the F_2 output. As far as we are aware the subjects studied by these authors even on a minimal niacin intake failed to develop pellagra or any other manifestation attributable to niacin deficiency. The F_2 output would seem to be a better criterion of the adequacy of niacin nutrition than a niacin assay of the diet.

Summary (1) Four subjects subsisted for a period of 3 months on a diet of purified vitamin-free foods furnishing a daily intake of 1.5-2.0 mg nicotinamide. (2) There were no symptoms or signs of nicotinic acid deficiency observed during this period. (3) The urinary output expressed as N-methylnicotinamide chloride showed no reduction suggestive of depletion of nicotinamide stores, a finding which suggests synthesis of the vitamin by the intestinal bacteria. (4) An attempt to inhibit bacterial synthesis of nicotinamide in the intestinal tract was not reflected by a drop in the urinary excretion of N-methylnicotinamide (F_2). (5) This suggests that nicotinamide synthesis in the intestinal tract was accomplished by bacteria which were not sensitive to sulfasuxidine in the doses used.

15326 P

Functional and Fatty Changes in Liver During Chronic Vitamin B Complex Deficiency*

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A decrease in hepatic function has been reported in dogs when a diet producing black-tongue is fed¹ or during an acute deficiency of the vitamin B complex.² Thus, vitamin B

complex may play a role in maintaining normal liver function. In such animals the decreased protein intake may also be a factor in producing hepatic dysfunction. The reduction of the protein intake of dogs to zero produces dye retention.³

In the present study a chronic deficiency of the whole vitamin B complex was produced. By using appropriate inanition controls the effect of the vitamin deficiency state

*Aided by a grant from Eli Lilly and Company, Indianapolis, Indiana, and the Fluid Research Fund of Yale University School of Medicine.

¹ Rhoads, C. P., and Miller, D. K., *J. Exp. Med.*, 1938, **67**, 463.

² Drill, V. A., Shaffer, C. B., and Lenthem, J. H., *Proc. Soc. Exp. Biol. and Med.*, 1943, **52**, 328.

³ Hough, V. H., Monahan, E. P., Li, T. W., and Freeman, S., *Am. J. Physiol.*, 1943, **139**, 642.

TABLE I
Food Intake and Liver Function of Chronic Yeast Deficient Dogs

41% casein (Exp 1)				
Weeks	Food intake		Bromsulphalein retention* %	Serum phosphatase* units
	Cal/sq M/hr	% of normal dogs		
0 5	46 63	65 103	2 6	2 5 4 5
6 10	42 49	64 100	2 3	3 5 4 0
11 20	32 47	37-74	3 5	2 0 5 0
21 30	15 35	23 45	3 5	2 5 5 0
31 39	17 35	29 55	3 5	2 2 6 0

20% casein (Exp 2)				
Weeks	Food intake		Bromsulphalein retention* %	Serum phosphatase* units
	Cal/sq M/hr	% of normal dogs		
0 5	50 83	86 120	3 4	2 0 4 0
6 10	22 51	31 69	3 16 (1)	2 5 4 0
11 20	18 44	34 68	3 18 (2)	3 0 6 0
21 27	37 58	44 86	4 7 (1)	4 0 7 5 (1)
28 31	34 45	60 71	2 19 (2)	2 5 6 5 (1)
31 33	25 34	49 60	12 60 (3)	4 0 10 0 (2)
34 37	22	29 46	3 10	2 0 4 0
38 42	26	34 48	3 7	2 0 5 0

* Upper limit of normal bromsulphalein retention, 6%, serum phosphatase 50 units
 Parentheses indicate number of dogs showing change from normal

on hepatic function was determined

Methods A total of 16 adult dogs received a synthetic diet containing either 41% or 20% casein[†]. The amount eaten during a 3-hour period each day was averaged for the week and calculated as calories consumed per square meter of surface area per hour. Three chronic vitamin B complex deficient dogs in each study received 0.1 g yeast 2019 per kilo per day. Three normal control and 2 inanition control dogs, used in each study, received 4.0 g type 3 yeast extract per day,[‡] which supplied a normal intake of B vitamins. Normal control dogs were used to measure normal fluctuations in food intake.

[†] Diet consisted of casein 20, lard, 21.5, bone ash, 2.6, salt mixture (Kerr), 1.3, and sucrose, 54.6%, plus 20 drops of oleum perecomorphum per kilo of diet. When the casein was increased to 41% the sucrose was correspondingly reduced.

[‡] Yeast 2019 contained per g: thiamine, 18.30 γ , riboflavin, 65 γ , pantothenic acid, 150 γ , nicotinic acid, 550 γ , pyridoxine, 40 γ , choline, 4 mg, inositol, 4 mg, and biotin, 2.5 γ . Type 3 yeast extract contained per g: thiamine, 900 γ , riboflavin, 210 γ , pantothenic acid, 450 γ , nicotinic acid, 40 mg, pyridoxine, 150 γ .

The authors wish to thank Mr. R. F. Light of the Fleischmann Laboratories for the analyzed yeast, and Dr. C. E. Bills of Merd Johnson and Company for the oleum perecomorphum.

Control inanition dogs were limited to the same number of calories, per square meter of surface area, consumed by the chronic B complex deficient animals the previous week. Liver function was studied by means of alkaline serum phosphatase⁴ and bromsulphalein⁵ (5 mg/kg.) determinations.

Results *Experiment 1* The chronic B complex deficient animals showed a progressive decrease in food intake which fell to 29-55% of normal (Table I). During the 39 weeks of low yeast supplement all groups of dogs maintained a normal bromsulphalein clearance and normal serum phosphatase.

Experiment 2 The chronic B complex deficient animals, receiving the same yeast intake as in Experiment 1 but with a 20% casein diet, also showed a decrease in food intake (Table I). Changes in bromsulphalein retention and serum phosphatase occurred in 2 of the 3 dogs before the 27th week (Table I). This finding is in contrast to the lack of changes in Experiment 1 during the same period, when a similar yeast intake was given but with a 41% casein diet.

On the 27th week the yeast intake of the deficient dogs was reduced by one-half. This

⁴ Bodansky, A., *J Biol Chem*, 1937, **120**, 167.

⁵ Rosenthal, S. M., and White, E. C., *J Am Med Assn*, 1925, **84**, 1112.

produced a further decrease in food intake. The bromsulphalein retention increased in all 3 dogs, and the serum phosphatase increased in 2 of the 3 dogs to 8 to 10 units/100 cc. Liver biopsies taken during the 32nd week showed a considerable amount of fat in all 3 B complex deficient dogs. Neither the inanition control nor the normal control dogs showed abnormal changes in liver function or fatty changes in the biopsy.

At the end of the 33rd week each B complex deficient dog was given 4 g of type 3 yeast extract per day and the food intake was limited to 22 calories/sq meter/hour, so that protein and total caloric intake was still below normal. One of the 3 dogs died during this period but the other 2 dogs showed a rapid return of bromsulphalein retention and serum phosphatase to normal. Liver

sections at the 42nd week showed either a definite decrease in fat or complete return of the fatty change to normal.

Summary A chronic vitamin B complex deficiency in dogs on a 41% casein diet produced a marked decrease in voluntary food intake, but liver function tests remained normal and liver sections showed no increase in fat. A similar chronic B complex deficiency using dogs on a 20% casein diet produced an abnormal liver function and fatty changes in liver biopsy specimens. These findings were absent in the inanition and normal control dogs. Therapy with yeast extract restored the liver function to normal and the fatty change in the liver decreased, even though protein and caloric intake were restricted.

15327

Role Played by Leucocytes, Platelets and Plasma Trypsin in Peptone Shock in the Dog

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We have previously studied the mechanism leading to the discharge of histamine and heparin from the liver in an anaphylaxis-like condition produced in dogs by the injection of extracts from *Ascaris lumbricoides*.^{1,2} On the basis of *in vitro* and *in vivo* experiments, performed in 84 dogs, we have concluded that the discharge of those substances constitutes the last step in a complex chain of reactions involving (1) clumping of leucocytes and platelets which will form micro-thrombi inside the liver capillaries and small vessels, (2) disintegration of platelets (as shown in Giemsa stained smears from

fragments of the liver) and also, in a less degree, of leucocytes in intimate contact with liver cells, (3) probable activation of a proteolytic enzyme, since platelets contain a kinase for plasma trypsin,³ and (4) the release of histamine and heparin by activated trypsin, since this enzyme is able to accomplish both effects.^{4,5}

All the steps of this chain of enzymatic and pharmacological reactions have been properly demonstrated, except activation of plasma trypsin during severe anaphylactic shock. When we attempted to estimate free trypsin in the plasma of animals, before and

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¹ Rocha e Silva, M., and Graña, A., *Arch Surg*, in press.

² Rocha e Silva, M., Porto, A., and Andrade, S. O., *Arch Surg*, in press.

³ Iyengar, N. K., *Indian J Med Res*, 1942, 30, 467.

⁴ Rocha e Silva, M., *Arch f exp Path und Pharmacol*, 1940, 19-1, 335.

⁵ Rocha e Silva, M., and Dragstedt, C. A., *Proc Soc Exp Biol and Med*, 1941, 48, 152.

TABLE 1
Variations in Prothrombin Time, Histamine, Platelets and Leucocytes After the Injection of Peptone in the Dog

No of the dog	Wt of the dog	Intensity of the shock	Prothrombin time			Histamine (g/ml)			Platelets (ml/100 ml)			Leucocytes (1000/mm ³)		
			Before	Min	Final	Before	Min	Final	Before	Min	Final	Before	Min	Final
P														
36	9	++	12'	120"	00	0.3	97	21	15	0	10	25.0	3.0	5.2
40	4	++	9	00	00	06	53	109	27	0	0	6.7	0.1	1.6
54	11	++	13	35	17"	0.5	58	41	70	0	0.5	12.9	2.6	2.9
51	9	++	10	80	55	1.6	50	11	18	0	0.8	8.9	2.8	4.6
43	13	++	15	35	210	0.1	15	11	20	0	1.2	5.7	1.5	2.3
50	10	++	11	00	35	1.1	109	99	22	0.7	1.2	5.0	1.1	2.1
59	7	++	11	11	13	0.6	41	06	15	0.8	35	9.3	2.8	6.1
44	15	++	11	11	10	1.2	15	12	65	0.3	4.2	12.1	2.1	13.3
47	22	++	9	9	8	1.0	25	20	90	0.5	19	18.3	5.7	13.8
33	5.5	++	9	9	9	0.1	11	03	35	0.5	25	17.3	3.3	5.2
52	13	++	11	22	11	0.8	35	15	65	0	10	15.9	2.9	19.2
37	9	++	10	12	9	0.5	23	13	50	0.5	15	11.7	6.1	8.5
39	9	++	10	10	10	1.3	17	11	45	1.2	15	9.5	9.3	9.1
39	8.5	+	11	13	13	0.3	19	06	70	25	35	13.5	6.3	10.3

Note: The animals 40P and 50P received 30 mg of heparin per kilo of body weight, before the injection of peptone. As seen in the table, this heparin injection did not prevent the drop in platelets and leucocytes. Upon the blood pressure, the effect of any was rather in aggravating one.

Legend: ++++, animal died; 20' to 60' without recovery; ++, incomplete recovery, without return to normal level; +, moderate fall in carotid blood pressure and return to normal in 20 to 30 min; +, fall in carotid blood pressure and return to normal level in <10 or 12 min.

produced a further decrease in food intake. The bromsulphalein retention increased in all 3 dogs, and the serum phosphatase increased in 2 of the 3 dogs to 8 to 10 units/100 cc. Liver biopsies taken during the 32nd week showed a considerable amount of fat in all 3 B complex deficient dogs. Neither the inanition control nor the normal control dogs showed abnormal changes in liver function or fatty changes in the biopsy.

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15327

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We have previously studied the mechanism leading to the discharge of histamine and heparin from the liver in an anaphylaxis-like condition produced in dogs by the injection of extracts from *Ascaris lumbricoides*.^{1,2} On the basis of *in vitro* and *in vivo* experiments, performed in 84 dogs, we have concluded that the discharge of those substances constitutes the last step in a complex chain of reactions involving (1) clumping of leucocytes and platelets which will form micro-thrombi inside the liver capillaries and small vessels, (2) disintegration of platelets (as shown in Giemsa stained smears from

fragments of the liver) and also, in a less degree, of leucocytes in intimate contact with liver cells, (3) probable activation of a proteolytic enzyme, since platelets contain a kinase for plasma trypsin,³ and (4) the release of histamine and heparin by activated trypsin, since this enzyme is able to accomplish both effects.^{1,5}

All the steps of this chain of enzymatic and pharmacological reactions have been properly demonstrated, except activation of plasma trypsin during severe anaphylactic shock. When we attempted to estimate free trypsin in the plasma of animals, before and

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³ Iwagiri, N. K., *Indian J Med Res*, 1942, 30, 467

⁴ Rocha e Silva, M., *Arch f exp Path and Pharmacol*, 1940, 194, 335

⁵ Rocha e Silva, M., and Dringstedt, C. A., *Proc Soc Exp Biol and Med*, 1941, 48, 152

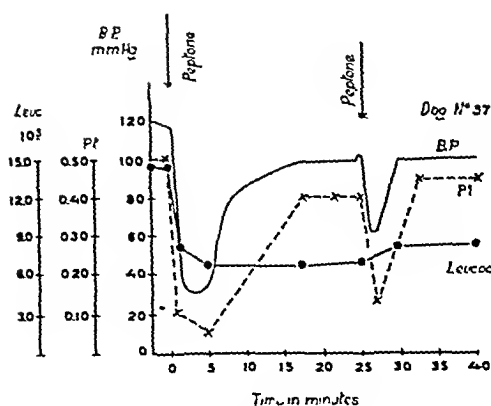


Fig. 2

Dog 37P. A first injection of peptone produced profound drop in blood pressure but a recovery almost to normal, after 20. A second injection produced a smaller effect and a quicker recovery. Platelets accompanied the curve of blood pressure, in a quite remarkable way.

the injection of the peptone the minimum being attained, as a rule, after 3 to 5 minutes following the injection. The data for prothrombin time are indicated as a rough measure of the discharge of antithrombin (heparin), since we have observed a certain parallelism between increases in prothrombin time and the amounts of heparin present. Additional information concerning the discharge of heparin in 4 of the animals employed can be obtained in Fig. 3, in which the results given by protamine test are indicated.

In cases of slight (+) or moderate (++) shock, the return of leucocytes and platelets was very rapid, accompanying the return to normal of carotid blood pressure. In those cases in which the shock was maximal, as carotid blood pressure fell rapidly to zero, platelets were down for the duration of the shock and did not return until death. Also the discharge of histamine and heparin was maximal in those cases. In the case of dog 51P the shock was particularly severe, with appreciable discharge of histamine and heparin the blood pressure was maintained at a low level for a long while and only slowly did incomplete recovery occur. As indicated in the table, platelets fell considerably and remained down at the end of the experiment. Of course, we cannot establish an absolutely

perfect parallelism between recovery and the capacity for platelets and leucocytes to return to their initial level. In the first place, when platelets are initially high, a partial return would not mean that the elements were destroyed somewhere in the body, since they can be retained in some unspecified organ structures, as occurs when one injects glycogen or any platelet-leucocyte agglutinating substance¹⁴. On the other hand, even when the leucocyte-platelet counts fully recover, there might occur some platelet destruction, since the returning elements might derive from previous depots in tissues which might not be concerned in the reaction. But, if one considers extreme cases, the parallelism indicated in Table I and Fig. 1 and 2, is striking enough to permit the conclusion that platelets and leucocytes take part in the mechanism of production of peptone shock.

Very important is the question as to where white elements segregate during peptone shock. From the perfusion experiments presented in another section of this paper it became very clear that the liver is able to retain those elements. But also *in vivo*, we have indications that those elements are concentrated in the portal circulation, since in many cases in which samples were taken at the trunk of the portal vein immediately after or before a similar sample was taken from femoral vein or inferior vena cava, leucocytes and platelets were enormously increased in the portal vein blood and in most cases there was a moderate but significant difference between those counts in portal and peripheral blood. In the case of dog 47P, for instance, the animal died with a leucocyte count of 4,500 per cu mm and a platelet volume of 0.10%, in a sample taken at inferior vena cava, while a sample taken simultaneously in the trunk of the portal vein showed a leucocyte count of 49,700 per cu mm and 0.50% of platelets. Another striking result was obtained in dog 58P, which died in 17 minutes, with leucocyte count of 1,600 and a platelet volume of 0.05%, while a simultaneous sample taken at the trunk of the portal vein showed 11,000 leucocytes

¹⁴ Rocha e Silva, M., Graña, A., and Porto, A., *Proc Soc Exp Biol and Med*, 1945 **59**, 57.

after the onset of the anaphylactic reaction, the only effect we have been able to detect was a rather drastic reduction in both free and total trypsin. We have interpreted this finding by assuming that the heparin which appears in blood stream would instantaneously block the action of activated trypsin, since heparin was shown in separate experiments to antagonize plasma trypsin.⁶ That this interpretation was correct, has been suggested by Jaques⁷ who has observed that addition of protamine to blood samples collected at the height of the anaphylactic shock brings about coagulation and, in many instances, a quick redissolution of the coagulum. Since fibrinolysis strongly suggests activation of plasma trypsin,^{8,9} this fact and those here presented constitute the demonstration that this enzyme might be concerned in the onset of anaphylactic and peptone shock.

Material and Methods The peptone used in those experiments was a proteose-peptone (Difco) containing small quantities of histamine, which amount was subtracted from the total, in experiments of liver perfusion, in the *in vivo* experiments, the histamine contained in the peptone injected was considered of minor significance for the final computation of the results. The heparin used was a Connaught preparation, containing 110 units per mg. The dogs were anesthetized with morphine (10 mg per kilo) plus Dial (Ciba) in the dose of 0.16 ml per kilo of body weight. When necessary, ether was used as a supplement. The *in vivo* experiments were performed by taking a tracing of the carotid blood pressure and collecting several blood samples before and at varying intervals after the injection of peptone. The samples were destined (a) for a duplicate estimation of histamine by Code's method,¹⁰ (b) for determination of prothrombin time by Quick's one stage method¹¹

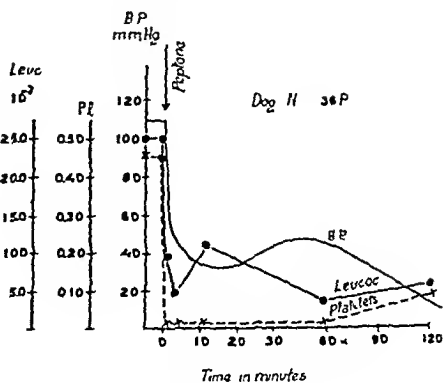


Fig 1

Dog 36P. Fatal shock. Variations in leucocytes, platelets and carotid blood pressure. Note that those variables went quickly to a very low level and remained so until death.

and fibrinogen, (c) for leucocyte counting, (d) for estimation of platelets volume by the method of the Van Allen's thrombocytocrit,¹² (e) for estimation of heparin by the protamine test, following the indications of Jaques and Waters.¹³ Data concerning fibrinogen are analyzed in a separate publication. Fibrinolysis was observed in the samples which clotted after addition of protamine, and the data indicated in the text refer to a period of 24 hours of observation. Perfusion experiments upon the isolated liver were performed according to the technic previously described.^{1,2}

Results In Table I and Fig 1 and 2 are indicated the variations in prothrombin time, histamine, leucocytes and platelets in 13 dogs submitted to peptone shock. At least 10 samples were taken at several intervals before and after the injection of 300 mg of peptone per kilo of body weight. From this considerable mass of data, those values were selected to show the initial level, the maximum or the minimum for each variable and also the final value, indicating the capacity of each animal to recover from initial changes. There was always a sharp decrease in leucocytes and platelets immediately after

⁶ Roehrer e Silva, M., and Andrade, S. O., *Science*, 1945, **102**, 670.

⁷ Jaques, L. B., personal communication.

⁸ Tagnon, H. J., *J. Lab. Clin. Med.*, 1942, **27**, 1119.

⁹ Ferguson, J. H., *Science*, 1943, **97**, 319.

¹⁰ Code, C. F., *J. Physiol.*, 1937, **89**, 257.

¹¹ Quick, A. J., *The Hemorrhagic Diseases and*

the Physiology of Hemostasis, 1942, C. C. Thomas, Springfield.

¹² Van Allen, C. M., *J. Lab. Clin. Med.*, 1926, **12**, 282.

¹³ Jaques, L. B., and Waters, E. T., *J. Physiol.*, 1941, **99**, 454.

a more or less extensive fibrinolysis in samples which clotted by adding protamine in suitable amounts. Therefore we decided to make systematic observations upon this phenomenon in peptone shock, and typical results are presented in Fig 3. When the animals received 2 or more injections of peptone, it was observed that fibrinolysis is maximal after the first injection, moderate after the second injection and absent after the third injection. It is obvious that this desensitizing effect occurring also with fibrinolysis, strongly suggests that the latter is concerned with the very mechanism of the shock. In control experiments, the *in vitro* addition of large amounts of peptone directly to blood samples failed to produce any fibrinolytic effect.

In discussing the results obtained with this "fibrinolytic protamine test," we might recall that Nolf¹⁵ had shown fibrinolysis occurring after peptone injection in the dog in those cases in which the liver was removed from circulation, or the circulation restricted to the upper part of the body, by ligating the inferior vena cava and thoracic aorta just above the diaphragm. Since heparin is discharged from the liver, it appears quite evident that shunting off the liver from circulation would bring experimental conditions for production of fibrinolysis quite similar to those obtained *in vitro* by adding an excess of protamine. Those facts substantiate the theory that activated plasma trypsin might constitute a final mediator for the discharge of histamine and heparin from liver cells since that enzyme is able to produce both effects, not only *in vivo*, but also in experiments of liver perfusion.²

Experiments of liver perfusion with peptone

In the previous experiments performed with ascaris extracts it became apparent that the simple contact of the material with liver cells, when Tyrode solution was used as perfusing fluid, is unable to discharge histamine and heparin. When total citrated blood was used, there occurred considerable retention of leucocytes and platelets and in one case, the discharge of histamine and anti-coagulant

was large enough to afford the conclusion that blood contains all the elements which are necessary to produce that discharge. Quite similar results were obtained by perfusing dogs' liver with peptone. When Tyrode solution was used, even large amounts of peptone were unable to produce any reduction in the perfusing flow or increase in the size of the organ. Also histamine and heparin did not appear in the perfusates, even after 30 to 40 minutes of perfusion with Tyrode + peptone. However when total heparinized blood containing 1 to 3 g of peptone was substituted for the Tyrode solution, immediately after the first portion of blood had passed, the flow was drastically reduced almost to zero (only a few drops passed from an initial velocity of 80 to 100 ml per minute) and the organ showed enormous increase in volume, almost doubling in a few minutes. Counting of leucocytes and platelets before and after the passage through the organ showed enormous reductions. In the case of the dog 60P, for instance, the platelets that were very high (115%) at the beginning dropped to 0.20 and 0.05% immediately after addition of peptone and passage through the liver. At the same time the leucocytes dropped from an initial level of 10,900 per cu mm to 1,000 and 900 per cu mm and those counts remained low for the duration of the experiment. Also the perfusion rate fell to a very low level (a few cu mm per minute) so that the perfusion could not proceed since the organ was cold and dark, indicating a complete lack of irrigation by the perfusing fluid. In the drainage fluid (obtained by making sharp incisions in the organ, after stoppage of the perfusion) the leucocytes rose again to a high level (17,950 per cu mm) and the platelets volume went up to 0.85%. As concerns the appearance of histamine and heparin in the perfusates when total blood was used as vehicle we have to emphasize that the amounts never were as high as should be expected from *in vivo* experiments. Also fibrinolysis occurring in the perfusates could be observed only occasionally and was not as striking as in *in vivo* experiment. From those facts we might conclude either that some extra-hepatic factor contributes to rein-

¹⁵ Nolf, P., *Medicine*, 1938, 17, 381

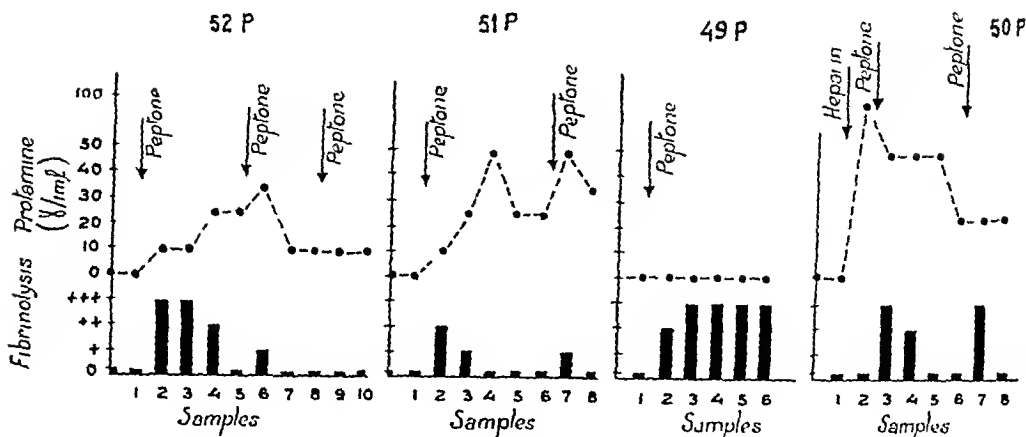


Fig. 1

Fibrinolysis and heparin discharge during peptone shock. The curves indicate the optimum amount of protamine to counteract the effect of the heparin present in samples taken at different stages of the shock. The indicated fibrinolysis was observed in the samples which clotted after addition of the optimum or higher amounts of protamine.

per cu mm and 0.90% of platelets. Similar results were obtained in quite a few cases and are definitely in accordance with the idea that the liver behaves like a filter for the white blood elements, during peptone shock.

The data presented in Table I show a definite relationship between the discharge of histamine and of anti-coagulant and the severity of the shock. Hence, fall in carotid blood pressure, drop in leucocytes and platelet volume, discharge of histamine and heparin, as well as the gross appearance of the liver by direct inspection, constitute the variables that must be considered in describing anaphylactic and peptone shock in the dog. All of them appear to run quite parallel and this fact affords considerable evidence pointing to the idea that they are fundamentally interconnected. Besides those 6 variables, we have to consider hemorrhages to the intestinal mucosa, leading to bloody diarrhea, and activation of plasma trypsin, as indicated by the observation of fibrinolysis occurring after addition of protamine, as will be considered in the next paragraph. We now have indications that the occurrence of hemorrhages to the intestinal mucosa might also be a consequence of the activation of a tryptic enzyme, since trypsin given to normal or heparinized dogs, can produce hemorrhages

in the intestinal tract, and this effect seems to depend upon a direct effect of the enzyme upon the vessels of that region, without very much relationship to the stasis and drop in carotid blood pressure.

Activation of plasma trypsin in peptone shock. As mentioned previously, estimation of plasma trypsin during anaphylactic shock in the dog showed that the changes were exactly the reverse of those we might expect from the assumption that disintegrated blood elements might activate this enzyme inside the liver parenchyme. There was consistently a drop in both free and total trypsin, somewhat correlated to the severity of the shock. This was interpreted as resulting from the discharge of heparin or any anti-tryptic heparin-like substance. In peptone shock, however, we had frequently observed many intriguing phenomena which could only be interpreted by assuming that plasma trypsin is actually activated. Sometimes, after the injection of peptone, the blood clotted very quickly (1' to 2') and in one instance it even clotted while pipetting into the tubes containing protamine (dog 49P), after a short while the clots redissolved. While doing those experiments, I received a note from Dr. L. B. Jaques, with the illuminating statement that in his experiments with the protamine test in anaphylactic shock, there frequently occurred

the children proved to be illegitimate

In determining to which of the 8 Rh types an individual belongs, tests are made with antisera for factors Rh₀, Rh' and Rh'' (Wiener⁶) Levine⁷ was the first to point out that Rh-negative blood is not merely characterized by its failure to clump in anti-Rh sera because such blood was agglutinated by an antiserum obtained by him from an Rh-positive mother of an erythroblastotic infant. The factor detected by Levine in Rh-negative blood seemed to be reciprocally related to Rh, and so he named this new factor Hr. In England, Race and Taylor⁸ under similar circumstances encountered a serum which also agglutinated all Rh-negative bloods but gave 80% positive reactions instead of 30% as reported by Levine. The British workers designated their factor as St after the first 2 letters of the patient's name. Wiener *et al*² showed that Hr and St were identical and pointed out that the difference in the number of positive reactions was due to the low titer of Levine's serum which therefore failed to react with heterozygous bloods.

As Race *et al*⁹ have shown, the Hr (or St) factor is present in the agglutinogens determined by the genes *Rh₂*, *Rh₀*, *Rh'* and *rh* but absent from the agglutinogens determined by genes *Rh₁* and *Rh'* (cf Wiener¹⁰). Therefore, the original or standard Hr factor is related to factor Rh' as M is related to N^{2,11}. To indicate this fact, we^{12,13} suggested to

designate the standard Hr factor simply as Hr'. This latter designation will become increasingly desirable in the future, especially since Fisher¹⁴ suggested that there are 3 factors in Rh-negative blood, each reciprocally related to one of the 3 Rh factors. To date we have studied 3 different Hr antisera and all of these (like the original Hr serum of Levine, and of Race *et al*) corresponded to anti-Hr' or standard anti-Hr. Mourant¹⁵ has recently encountered a serum giving reactions corresponding to those predicted for anti-Hr'', but to date no serum has been found corresponding to anti-Hr₀.

Of the 3 theoretically possible anti-Hr sera, only anti-Hr' (or standard anti-Hr) is generally available at present. Fortunately, standard anti-Hr serum is the most valuable because it makes possible the subdivision of the most common Rh type, type Rh₁. The use of anti-Hr serum together with the 3 anti-Rh sera enhances considerably the practical value and theoretical interest of heredity studies on the Rh blood types. Race *et al*^{16,17} and Stratton¹⁸ have published family studies on the Rh blood types in which Hr tests were also performed.

The purpose of this report is to present our recent experiences in family studies on the hereditary transmission of the Rh blood types and the standard Hr factor. We shall take this opportunity also to introduce some improvements in the nomenclature made possible by recent advances in our knowledge of this subject.

Materials and Methods The blood samples tested in our heredity studies were derived from clinical material sent to us for diagnostic Rh tests in cases where it was suspected that one or more of the children in a family had congenital hemolytic disease (erythroblastosis fetalis). This accounts for the abnormal distribution of the Rh blood

³ Sonn, E. B., and Wiener, A. S., *J. Hered.*, 1945, **36**, 301.

⁴ Wiener, A. S., and Sonn, E. B., *J. Lab. and Clin. Med.*, 1945, **30**, 395.

⁵ Wiener, A. S., *Proc. Soc. Exp. Biol. and Med.*, 1943, **54**, 316.

⁶ Wiener, A. S., *Am. J. Clin. Path.*, 1945, **15**, 106.

⁷ Levine, P., *J. Ped.*, 1943, **23**, 656.

⁸ Race, R. R., and Taylor, G. L., *Nature*, 1943, **152**, 300.

⁹ Race, R. R., Taylor, G. L., Cappell, D. T., and McFarlane, M. N., *Nature*, 1944, **153**, 52.

¹⁰ Wiener, A. S., *J. Am. Med. Assn.*, 1945, **127**, 294.

¹¹ Levine, P., *Science*, 1945, **102**, 1.

¹² Wiener, A. S., and Sonn, E. B., *Ann. N. Y. Acad. Sci.*, in press.

¹³ Wiener, A. S., *Science*, 1945, **102**, 479.

¹⁴ Fisher, R. A., cited by Race, R. R., *Nature*, 1944, **153**, 771.

¹⁵ Mourant, E., *Nature*, 1945, **155**, 542.

¹⁶ Race, R. R., Taylor, G. L., Ihm, E. M., and Prior, A. M., *Ann. Eug.*, 1944, **12**, 206.

¹⁷ Race, R. R., Taylor, G. L., Ihm, E. M., and Dobson, A. M., *Ann. Eug.*, 1945, **12**, 261.

¹⁸ Stratton, F., *Ann. Eug.*, 1945, **12**, 250.

force activation of plasma trypsin as it occurs *in vivo*, or that the conditions of the perfusion experiments only exceptionally reproduce the conditions of the phenomenon as it occurs *in vivo*. Although those possibilities will be considered in future experiments, we can draw certain conclusions from the perfusion experiments concerning the mechanism of the preliminary phases of the shock, namely constriction of hepatic veins and retention of leucocytes and platelets inside the organ, these constituting the main factors for the enlargement of the organ and stasis in portal region. The importance of this retention was further proved in those experiments in which Tyrode was used as perfusing fluid and also in 2 other experiments in which the blood used for perfusion was extremely poor in white blood elements. In those cases, there was very slight diminution of flow, in contrast with the almost complete stoppage of the circulation when the white blood elements were high enough in the perfusing heparinized blood.

Conclusions From the results described in this paper, it appears evident that peptone produces shock in the dog by a mechanism very similar to that leading to anaphylactic shock. Participation of the white blood elements (leucocytes and platelets) in the discharge of histamine and heparin from liver cells is definitely suggested by the fact that

the severity of the shock is closely connected with the incapacity of those blood elements to return to blood stream, and also by experiments with isolated liver, in which it became apparent that peptone is unable by itself to release those substances from liver cells, and that the presence and retention of blood elements (leucocytes and platelets) is an indispensable step leading to the discharge of those substances. That the final mediator for the release of these substances is activated plasma trypsin is strongly suggested by the fact that platelets contain a kinase for plasma trypsin and that there actually occurs activation of this enzyme, as shown by fibrinolysis occurring after the injection of peptone. Also the fact that fibrinolysis is maximal after the first injection of peptone, moderate after a second injection and minimal or absent after a third injection, constitutes strong evidence that activation of plasma trypsin is actually concerned in the mechanism of production of the shock and that desensitization which occurs in those kinds of shock is probably dependent upon exhaustion of the capacity for activation of plasma trypsin. Experiments upon those lines are being actively conducted in this laboratory.

We have to thank Dr. Wilson T. Beraldo, of the Faculty of Medicine of São Paulo, for help given in the last phases of this work, and Mr. Jaime Faria, who made leucocyte and platelet estimations.

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Heredity of Rh Blood Types * V Improved Nomenclature, Additional Family Studies with Special Reference to Hr

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In preceding papers of this series¹⁻⁴ data have been presented on the heredity of the Rh blood types in a total of 197 families

with 463 children. Only 2 apparent exceptions to Wiener's theory⁵ of 6 major allelic genes were encountered, and in both cases

* Aided by a grant from the United Hospital Fund of New York City.

¹ Wiener, A. S., Sonn, E. B., and Belkin, R. B.,

J. Exp. Med., 1944, **79**, 235.

² Wiener, A. S., Davidsohn, I., and Potter, E. L., *J. Exp. Med.*, 1945, **81**, 63.

TABLE II
List of Family Material

Family No	Father	Mother	Children	
1	OMRh ₂ Hr +	A ₂ MRh — Hr +	A ₁ MRh ₂ Hr + ♀	
2	ONRh ₁ Hr —	A ₁ MRh — Hr +	OMNRh ₁ Hr + ♀	
3	A ₁ NRh ₁ Hr +	OMNRh ₁ Hr +	ONRh ₁ Hr + ♀	ANRh ₁ Hr — ♂
4	OMNRh ₁ Hr —	A ₁ MRh — Hr +	A ₁ MNRh ₁ Hr + ♂	A ₁ MNRh ₁ Hr + ♀
			OMNRh ₁ Hr + ♀	OMRh ₁ Hr + ♂
5	OMRh ₁ Rh ₂ Hr +	BMNRh — Hr +	BMRh ₂ Hr +	
6	OMNRh ₁ Hr —	OMRh — Hr +	OMRh ₁ Hr +	
7	BMNRh ₁ Hr —	BMNRh — Hr +	BMNRh ₁ Hr + ♀	BNRh ₁ Hr + ♂
8	A ₁ MNRh ₁ Hr —	ONRh ₁ Hr +	A ₁ MNRh ₁ Hr +	
9	OMNRh ₁ Hr +	OMRh — Hr +	OMRh ₁ Hr + ♂	
10	A ₁ BMNRh ₁ Hr —	A ₁ MNRh — Hr +	A ₁ NRh ₁ Hr + ♂	
11	A ₁ MRh ₁ Hr —	A ₁ MNRh — Hr +	A ₁ MRh ₁ Hr + ♀	A ₁ MRh ₁ Hr + ♀
12	A ₁ BNRh ₁ Hr +	OMNRh — Hr +	BMNRh ₁ Hr + ♂	
13	OMRh ₂ Hr +	A ₂ MNRh — Hr +	A ₂ MNRh ₀ Hr + ♂	OMNRh ₂ Hr + ♀
			OMNRh ₂ Hr + ♂	
14	A ₂ BMNRh ₁ Hr +	ONRh ₁ Rh ₂ Hr +	BMNRh ₁ Hr +	
15	OMNRh ₁ Rh ₂ Hr +	A ₁ BMNRh — Hr +	BMRh ₁ Hr + ♂	BMNRh ₂ Hr + ♀
			BMRh ₂ Hr + ♂	
16	OMNRh ₁ Hr —	A ₂ MRh — Hr +	A ₂ MRh ₁ Hr + ♀	
17	A ₁ MRh ₁ Rh ₂ Hr +	A ₂ MNRh — Hr +	A ₁ MNRh ₁ Hr + ♀	A ₁ MNRh ₁ Hr + ♂
18	OMNRh ₂ Hr +	OMRh ₁ Rh ₂ Hr +	OMNRh ₁ Hr + ♀	OMRh ₁ Hr +
19	A ₁ NRh ₁ Rh ₂ Hr +	OMNRh ₁ Rh ₂ Hr +	OMNRh ₁ Rh ₂ Hr + ♀	
20	A ₁ MNRh — Hr +	A ₁ MNRh ₁ Hr —	A ₂ MNRh ₁ Hr —	
21	A ₁ BNRh ₁ Rh ₂ Hr +	OMRh ₂ Hr +	BMNRh ₁ Hr +	
22	OMRh ₁ Hr —	A ₁ NRh ₁ Hr —	OMNRh ₁ Hr — ♂	
23	A ₁ NRh ₀ Hr +	OMNRh ₁ Rh ₂ Hr +	A ₁ NRh ₁ Hr + ♀	
24	OMNRh ₁ Rh ₂ Hr +	A ₁ MRh ₁ Hr +	OMRh ₁ Rh ₂ Hr + ♂	A ₁ MNRh ₁ Rh ₂ Hr + ♂
			A ₁ MRh ₁ Hr — ♂	
25	A ₂ NRh ₁ Hr +	A ₁ BMNRh — Hr +	A ₁ MNRh ₁ Hr + ♀	BNRh ₁ Hr + ♂
26	BNRh — Hr +	OMNRh ₁ Hr +	BMNRh — Hr + ♂	BNRh ₁ Hr + ♂
27	BNRh ₁ Hr +	A ₁ NRh ₁ Hr +	ONRh ₁ Hr — ♂	ONRh ₁ Hr + ♀
28	OMNRh ₁ Hr —	OMRh ₁ Hr +	OMRh ₁ Hr —	
29	A ₁ MNRh ₁ Rh ₂ Hr +	A ₁ MNRh — Hr +	A ₁ NRh ₂ Hr + ♀	
30	A ₁ MRh ₁ Hr —	A ₁ MNRh ₁ Hr +	A ₁ MRh ₁ Hr + ♀	
31	A ₁ MNRh ₂ Hr +	A ₁ MNRh ₁ Hr +	ONRh — Hr + ♂	
32	OMRh ₁ Hr +	A ₁ MNRh — Hr +	A ₁ MRh ₁ Hr + ♀	A ₁ MRh ₁ Hr + ♂
33	A ₁ MRh ₁ Rh ₂ Hr +	A ₁ MRh ₁ Hr +	A ₁ MRh ₂ Hr + ♀	
34	A ₁ MNRh ₁ Hr +	OMRh ₁ Hr —	OMNRh ₁ Hr — ♀	ARh ₁ Hr +
35	OMNRh ₁ Hr +	OMNRh ₁ Hr +	OMNRh ₁ Hr + ♀	OMNRh — Hr + ♀
36	OMNRh ₁ Hr —	BMNRh — Hr +	BNRh ₁ Hr + ♂	
37	A ₂ BMNRh ₁ Rh ₂ Hr +	A ₁ MNRh — Hr +	A ₁ BMNRh ₂ Hr + ♀	BMNRh ₁ Hr + ♂
38	A ₁ MNRh ₁ Rh ₂ Hr +	A ₁ NRh — Hr +	A ₁ MNRh ₂ Hr + ♂	
39	OMNRh ₁ Hr —	A ₂ BNRh — Hr +	A ₂ BMNRh ₁ Hr + ♀	A ₂ Rh ₁ Hr + ♂
40	A ₂ MNRh ₁ Hr +	OMNRh ₁ Hr +	OMNRh ₁ Hr + ♂	OMNRh ₁ Hr + ♂
41	A ₁ MNRh ₁ Hr +	ONRh ₁ Hr +	A ₁ NRh ₁ Hr — ♀	
42	BMNRh ₁ Rh ₂ Hr +	ONRh ₁ Hr —	BMNRh ₁ Hr — ♂	
43	A ₁ BMNRh — Hr +	OMRh — Hr +	BMNRh — Hr +	
44	A ₁ MNRh — Hr +	OMRh — Hr +	OMRh — Hr + ♂	
45	BMNRh ₁ Hr +	A ₂ MRh — Hr +	A ₂ MRh — Hr + ♀	A ₂ BMRh ₀ Hr + ♀
46	OMNRh ₁ Rh ₂ Hr +	OMRh — Hr +	OMRh ₂ Hr + ♀	OMRh ₁ Hr + ♀
47	A ₁ MNRh ₁ Hr +	ONRh — Hr +	A ₁ NRh — Hr + ♀	ONRh — Hr +
48	BMRh ₁ Hr —	OMNRh — Hr +	BMNRh ₁ Hr + ♂	
49	OMNRh ₁ Hr —	OMNRh — Hr +	OMNRh ₁ Hr +	
50	OMNRh ₂ Hr +	A ₁ MRh ₁ Rh ₂ Hr +	OMRh ₂ Hr + ♂	
51	OMRh ₁ Hr +	A ₁ MNRh — Hr +	OMNRh ₁ Hr + ♀	OMNRh ₁ Hr + ♂
52	OMNRh ₁ Hr +	A ₁ NRh ₁ Hr +	ONRh ₁ Hr + ♂	
53	A ₂ MRh ₂ Hr +	A _{1,2} MNRh — Hr +	A ₂ MNRh — Hr + ♂	OMNRh ₂ Hr + ♀
54	OMRh ₁ Hr +	A ₂ BMNRh ₁ Hr +	A ₂ MRh — Hr + ♂	
55	BMRh ₁ Hr +	OMNRh ₁ Hr +	BMRh ₁ Hr + ♀	OMRh ₁ Hr + ♀
56	A ₂ MNRh ₂ Hr +	OMNRh ₁ Hr +	A ₂ MRh — Hr + ♀	A ₂ MNRh — Hr + ♀
			ONRh ₁ Hr + ♀	
57	OMNRh ₁ Hr +	OMNRh — Hr +	OMNRh ₁ Hr + ♂	OMNRh — Hr + ♀
58	A ₂ MNRh ₂ Hr +	A ₁ MRh Hr +	A ₁ MRh ₂ Hr + ♂	
59	OMRh ₁ Rh ₂ Hr +	A ₁ MNRh — Hr +	A ₁ MRh ₂ Hr + ♂	

TABLE I
The Rh Blood Types and Subtypes *

Rh blood types	Reactions with ser if			Genotypes	Reactions with ser if		Rh subtypes	Distribution (%) among 645 Caucasian in N Y C
	Anti Rh'	Anti Rh''	Anti Rh ₀		Anti Hr'	Anti Hr''		
rh	Neg	Neg	Neg	rr	Pos	Pos		12.9
Rh'	Pos	Neg	Neg	R'R'	Neg	Pos	Rh'Rh'	0.1†
				R'r	Pos	Pos	Rh'rh	0.9‡
				R''R''	Pos	Neg	Rh''Rh''	0.04‡
Rh''	Neg	Pos	Neg	R'r	Pos	Pos	Rh''rh	0.5‡
Rh'Rh''	Pos	Pos	Neg	R''R''	Pos	Pos		0.1‡
rh ₀	Neg	Neg	Pos	r ⁰ r ⁰	Pos	Pos		1.9
				r ⁰ r	Pos	Pos		
				R ¹ R ¹	Neg	Pos	Rh ₁ Rh ₁	20.2
Rh ₁ (Rh' ₀) Pos		Neg	Pos	R ¹ R'				
				R ¹ r	Pos	Pos	Rh ₁ rh	33.5
				R ¹ r ⁰				
				R' ⁰ r ⁰				
				R ² R ²	Pos	Neg	Rh ₂ Rh ₂	3.1‡
Rh ₂ (Rh'' ₀) Neg		Pos	Pos	R ² R''				
				R ² r	Pos	Pos	Rh ₂ rh	12.2‡
				R ² r ⁰				
				R''r ⁰				
				R ¹ R ²	Pos	Pos		14.4
Rh ₁ Rh ₂	Pos	Pos	Pos	R R ²				
				R R ¹				
Others‡§								0.5

* Does not include Rh₃ or the intermediate Rh blood types, or the reactions with the hypothetical serum anti Hr₀.

† Anti Rh₀ is the standard anti Rh serum (85% positive), anti Hr' is the standard anti Hr serum (80% positive).

‡ These frequencies were calculated on the basis of the 6 gene theory. All other frequencies given were actually observed. All 645 bloods were tested with anti Rh', anti Rh'', anti Rh₀ and anti Hr' sera, anti Hr'' serum was not available to us.

§ There were 3 bloods giving atypical reactions, namely, one of type Rh₁Rh₂ and 2 intermediates

types among the parents, in particular the high percentage of Rh-negative mothers. A random series of blood samples from male professional blood donors was also examined, for use as an index of the distribution of the Rh blood types among Caucasian individuals in New York City. With only a few exceptions, all blood samples were obtained by venipuncture, 5 cc of blood were mixed with powdered potassium and ammonium oxalate in a small test tube. The tubes containing the oxalate powder were prepared in the same way as for the Wintrobe-Landsberg¹⁹ sedimentation test.

The tests were carried out within 24 to 48 hours after the blood was drawn, the blood samples being kept in the refrigerator in the interim. Blood group, subgroup and M-N tests were performed on every specimen by

the well-slide method as described elsewhere²⁰. For the Rh and Hr tests, all blood suspensions were washed once with saline solution and the tests were set up in small tubes as already described.^{1,21}

Nomenclature The recent work with anti-Hr' and anti-Hr'' sera makes desirable certain changes and improvements in the nomenclature. Naturally such changes should be kept to an absolute minimum, and it must be emphasized that the recent results have merely added to our knowledge and not changed our previous conclusions. The improved nomenclature is presented in Table I.

Firstly, in regard to the Rh blood types, it is proposed to substitute the simple design-

¹⁹ Wintrobe, A. S., *Blood Groups and Transfusion*, 2nd ed., C. C. Thomas, Springfield, Ill., 1947.

²¹ Wiener, A. S., Zepeda, J. P., Sonn, L. B., and Polak, H. R., *J. Exp. Med.*, 1947, **81**, 559.

²⁰ Wintrobe, M. M., and Landsberg, J. W., *Am. J. Med. Sci.*, 1935, **180**, 102.

TABLE III
Summary of Family Material of Table II

Mating	No of families	No of children of types						Totals
		rh	Rh ₁	Rh ₂	Rh ₁ Rh ₂	rh ₀	Rh'	
rh × rh	3	4	0	0	0	0	0	4
rh × Rh ₁	33	9	49	0	0	2	0	60
rh × Rh ₂	7	1	0	10	0	1	0	12
rh × Rh ₁ Rh ₂	17	0	12	15	0	0	0	27
rh × rh ₀	1	0	0	0	0	1	0	1
Rh ₁ × Rh ₁	18	2	24	0	0	0	0	26
Rh ₁ × Rh ₂	7	4	5	2	0	0	0	11
Rh ₁ × Rh ₁ Rh ₂	3	0	6	2	5	0	0	13
Rh ₁ × rh ₀	1	1	0	0	0	0	0	1
Rh ₁ × Rh'	4	0	2	0	0	0	2	4
Rh ₂ × Rh ₁ Rh ₂	6	0	4	4	0	0	0	8
Rh ₁ Rh ₂ × Rh ₁ Rh ₂	2	0	2	1	1	0	0	4
Rh ₁ Rh ₂ × rh ₀	1	0	1	0	0	0	0	1
Rh ₂ × Rh'	1	0	0	1	0	0	0	1
Totals	109	21	105	35	6	4	2	173

nation "rh"[†] for the more cumbersome "Rh negative". The small "r" is used to indicate that type rh is recessive to the other Rh blood types. For type rh₀ the capital letter is no longer used, in order to indicate that this type has the same position in relation to types Rh₁, Rh₂ and Rh₁Rh₂ as type rh has in relation to types Rh', Rh'' and Rh'Rh''.

When the reactions of the anti-Hr' serum is taken into account, types Rh₁ and Rh' are both subdivided into 2 types. Only bloods with a double dose of the Rh' factor are Hr' negative, and to indicate this Hr'-negative blood of type Rh₁ is designated as subtype Rh₁Rh₁ and the corresponding subtype of type Rh' is designated Rh'Rh'. Blood of type Rh₁ reacting with Hr' anti-serum is designated as subtype Rh₁rh, the corresponding subtype of type Rh' is designated Rh'rh. With the aid of anti-Hr'' serum similar subtypes can be distinguished in types Rh₂ and Rh''. The advantages of the names for the subtypes proposed by us are their simplicity and the lack of ambiguity so that they cannot be confused with the names of the 8 Rh blood types or the genotypes.

[†] In giving the names of the Rh blood types verbally, it is not necessary to use the expressions "large Rh" or "small Rh" as the case may be, but merely to say "Rh," because the distinguishing subscripts and superscripts for the various types remove any danger of ambiguity.

Our previous designations²² for the 6 standard Rh genes have been criticized because the use of the subscripts for some of the allelic genes does not conform with the practice of geneticists. Another possible objection is that the use of the same symbol for the gene and corresponding agglutिनogen could cause uncertainty in some cases as to whether the genotype or phenotype was intended, even though italics is used for the gene symbols. These objections are both met by the new designations of the genes in which only superscripts are used and the letter "h" has been dropped as suggested by British workers[‡]. Thus, the new designations have the advantages of extreme simplicity, lack of ambiguity and conformity with genetic nomenclature.

The omission of the "h" from the designations of the genes makes it possible to use without danger of confusion the symbols *Rh* and *rh* when discussing the heredity of the Rh factor in general (factor Rh₀), for example, when presenting the theory of Land-

²² Wiener, A. S., *Science*, 1944, 99, 532.

[‡] The new simplified gene symbols, except for *rh*, are the same as those approved by the majority of the members of the American Committee on Nomenclature of Blood Group Genes (W. C. Boyd, chairman, Th. Dobzhansky, P. Levine, L. H. Snyder, H. H. Strandkor and A. S. Wiener) as well as the British Committee (R. A. Fisher, E. B. Ford, K. Mather and R. R. Race).

TABLE II (Continued)

Family No	Father	Mother	Children	
60	BMRh ₁ Hi+	ONRh—Hr+	OMNRh ₁ Hi+♂	OMNRh ₁ Hi+♀
61	A ₁ MNRh ₁ Hr+	A ₁ BMNRh—Hr+	A ₁ BMNRh—Hr+	
62	OMRh ₁ Hr—	BMRh ₁ Hr—	OMRh ₁ Hr—♂	
63	OMRh ₁ Hr—	OMRh—Hr+	OMRh ₁ Hr+♂	OMRh ₁ Hr+♀
			OMRh ₁ Hr+♀	OMRh ₁ Hr+♀
			OMRh ₁ Hr+♀	OMRh ₁ Hr+♂
			OMRh ₁ Hr+♀	
64	OMNRh ₁ Rh ₂ Hr+	A ₂ MNRh—Hr+	OMNRh ₂ Hr+♂	A ₂ MNRh ₂ Hr+♀
65	A ₁ MNRh ₁ Hr—	OMRh—Hr+	A ₁ MRh ₁ Hr+♂	
66	A ₁ MNRh ₂ Hr+	A ₁ MNRh—Hr+	OMNRh ₂ Hr+♂	
67	A ₁ NRh ₁ Hi+	OMNRh ₁ Hr—	A ₁ MNRh ₁ Hr—♀	
68	BNRh ₁ Rh ₂ Hr+	ONRh ₁ Rh ₂ Hr+	BNRh ₁ Hr—♂	ONRh ₁ Hr—♂
			BNRh ₂ Hr+♂	
69	BMRh ₁ Rh ₂ Hr+	A ₁ NRh—Hr+	OMNRh ₂ Hr+♂	A ₁ BMNRh ₁ Hr+♂
70	OMNRh ₁ Rh ₂ Hr+	A ₁ MNRh ₁ Hr+	A ₁ MRh ₁ Hr—♂	A ₁ MNRh ₁ Rh ₂ Hr+♂
			OMRh ₁ Rh ₂ Hr+♂	
71	A ₁ NRh ₁ Rh ₂ Hr+	OMRh ₁ Hr+	A ₁ MNRh ₁ Hr—	
72	A ₁ NRh ₁ Hr+	OMRh ₁ Hr—	OMNRh ₁ Hr+♂	A ₁ MNRh ₁ Hr+
73	A ₁ BNRh ₁ Rh ₂ Hr+	OMRh ₂ Hr+	BMNRh ₁ Hi+	
74	OMRh ₁ Rh ₂ Hr+	A ₁ MRh—Hr+	A ₁ MRh ₂ Hr+♂	
75	OMNRh ₂ Hr+	A ₁ MRh—Hr+	A ₁ MNRh ₂ Hr+♂	
76	ONRh ₁ Hr+	OMNRh ₂ Hr+	ONRh ₂ Hr+♂	OMNRh ₁ Hi+♂
			OMNRh ₁ Hr+♂	
77	A ₁ MRh ₁ Rh ₂ Hr+	A ₁ NRh ₁ Hr—	OMNRh ₁ Rh ₂ Hr+	
78	OMRh ₂ Hr+	BMRh—Hr+	OMRh ₂ Hr+♂	
79	BNRh ₁ Hr+	BMRh ₁ Hr+	BMNRh ₁ Hr—♀	
80	OMRh ₁ Rh ₂ Hr+	ONRh—Hr+	OMNRh ₂ ♂	
81	OMNRh ₁ Hr+	A ₂ MNRh—	A ₂ MRh—♂	A ₂ MRh ₁ ♀
			A ₂ MNRh ₁ ♀	A ₂ MNRh ₁ ♀
			A ₂ NRh—♀	
82	ONRh ₁ Hr—	A ₁ MNRh—Hr+	OMNRh ₁ ♀	A ₁ MNRh ₁ ♀
83	A ₂ MNRh ₁ Rh ₂	A ₂ MRh—	A ₂ MNRh ₂ Hr+♂	
84	A ₁ MNRh ₁ Hr—	A ₁ MNRh—Hr+	OMNRh ₁ ♀	A ₁ MRh ₁ Hr+♀
85	OMRh ₁ Rh ₂	A ₁ MNRh—	A ₁ MRh ₁ Hr+♂	A ₁ MRh ₁ Hr+♀
			A ₂ MRh ₁ Hr+♀	A ₂ MNRh ₁ Hr+♂
86	BMNRh ₁ Hr+	OMNRh ₁ Rh ₂ Hr+	BMNRh ₁ Hr—♂	BMNRh ₁ ♀
87	A ₁ MNRh ₁ Hr—	OMRh—Hr+	A ₁ MNRh ₁ ♀	
88	A ₁ NRh ₁ Hr+	OMNRh ₂	A ₁ NRh ₂ ♂	
89	A ₁ NRh ₂	A ₁ MNRh ₁ Rh ₂	A ₁ MNRh ₂ ♀	A ₁ NRh ₁ ♂
90	A ₁ MRh ₁	OMNRh—	OMRh—♂†	A ₂ MRh ₁ ♂†
91	BMNRh ₁	A ₁ MRh—	A ₁ MRh ₂	
92 †	BMRh—	A ₂ BMRh—	BMRh—♂	A ₂ BMRh—♂
93 †	A ₂ MRh ₁	BMRh ₂	A ₂ BMRh—♀	
94	OMNRh ₁ Rh ₂	ONRh—	ONRh ₁ ♀	
95	OMRh ₁	A ₁ MRh ₂	OMRh—	
96	A ₁ MRh—	ONRh ₁	A ₁ MNRh—	
97	ONRh ₁ Rh ₂	OMNRh—	OMNRh ₁ ♂	
98	A ₁ MNRh ₁	OMRh ₂	A ₁ MRh ₁	
99	OMRh ₂	A ₁ MRh—	A ₁ MRh ₂ ♀	
100	A ₁ MNRh ₂	A ₁ MRh ₁	A ₁ MRh ₁ ♀	
101	BMNRh ₁	A ₁ BMRh—	BMRh ₁ ♀	
102	A ₁ MRh ₁	A ₂ NRh ₁	A ₂ MNRh ₁ ♂	
103	A ₁ MNRh ₁	A ₁ NRh ₁	A ₁ NRh ₁ ♀	A ₁ MNRh ₁
104	A ₁ MNRh ₁ Rh ₂	A ₁ MNRh—	A ₁ NRh ₂	
105	OMRh ₂	OMRh—	OMRh ₂ ♂	OMRh ₂ ♀
			OMRh ₂ ♂	
106	A ₁ MRh ₁	BMNRh—	OMNRh ₁ ♂‡	OMNRh ₁ ♂§
107	BMRh ₁ Rh ₂	A ₁ MNRh ₂	BMRh ₂ ♀	
108	OMRh ₁	A ₁ MRh—	OMRh ₁	
109	A ₁ MNRh ₁	A ₁ MNRh—	OMNRh ₁ ♂	OMRh ₁ ♀

* First child in families 39 and 45 from former marriage of wife

† Stillborn twins

‡ Daughter in family 93 same as mother in family 92

§ Twins

TABLE V
Summary of Families on Which Hr Tests Were Done

Mating	No of families	No of children of types (or subtypes)								Totals
		rh	Rh ₁ rh	Rh ₁ Rh ₁	Rh ₂	Rh ₁ Rh ₂	rh ₀	Rh'rh	Rh'Rh'	
rh × rh	2	2	0	0	0	0	0	0	0	2
rh × Rh ₁ rh	10	5	11	0	0	0	1	0	0	17
rh × Rh ₁ Rh ₁	13	0	24	0	0	0	0	0	0	24
rh × Rh ₂	5	1	0	0	6	0	1	0	0	8
rh × Rh ₁ Rh ₂	11	0	6	0	12	0	0	0	0	18
rh × rh ₀	1	0	0	0	0	0	1	0	0	1
Rh ₁ rh × Rh ₁ rh	8	2	7	3	0	0	0	0	0	12
Rh ₁ rh × Rh ₁ Rh ₁	6	0	5	3	0	0	0	0	0	8
Rh ₁ Rh ₁ × Rh ₁ Rh ₁	2	0	0	2	0	0	0	0	0	2
Rh ₁ rh × Rh ₂	3	3	3	0	1	0	0	0	0	7
Rh ₁ rh × Rh ₁ Rh ₂	6	0	0	4	2	4	0	0	0	10
Rh ₁ Rh ₁ × Rh ₁ Rh ₂	2	0	0	1	0	1	0	0	0	2
Rh ₁ rh × Rh'rh	2	0	0	0	0	0	0	1	1	2
Rh ₁ Rh ₁ × Rh'rh	2	0	1	1	0	0	0	0	0	2
Rh ₂ × Rh ₁ Rh ₂	4	0	3	0	2	0	0	0	0	5
Rh ₂ × Rh'rh	1	0	0	0	1	0	0	0	0	1
Rh ₁ Rh ₂ × Rh ₁ Rh ₂	2	0	0	2	1	1	0	0	0	4
rh ₀ × Rh ₁ Rh ₂	1	0	1	0	0	0	0	0	0	1
Totals	81	13	61	16	25	6	3	1	1	126

on the other hand, in 10 matings rh × Rh₁rh with 17 children there were as many as 5 children of type rh and one child of type rh₀. The most unusual case is family No 79. Here the father belonged to subtype Rh₁rh, the mother to subtype Rh'rh, while the daughter belonged to subtype Rh'Rh'. As shown in Table I, individuals of subtype Rh₁rh may belong to any one of the 3 possible genotypes, R'r, R'¹r⁰ or R'⁰. In order for the couple in question to have a child of subtype Rh'Rh', it is evident that the father must belong to genotype R'⁰.

Another interesting case is family No 45. The mother belonged to type rh, her husband to type Rh₁rh, the older daughter to type rh and the younger daughter to type rh₀. Obviously the husband could not be the father of both daughters so we concluded that the first child was probably the result of a former marriage. When we called the mother back for a private interview she told us that such was indeed the case and that she had not mentioned it before because she did not want to discuss the matter in the presence of her daughters.

Statistical Analysis In Table I is given the distribution of the Rh blood types and subtypes in a series of 645 Caucasians in

New York City. Since anti-Hr'' serum was not available to us, the percentages of the subtypes of types Rh₂ and Rh'' had to be computed on the basis of the 6-gene theory, but all the other frequencies listed were actually observed. In our experience to date we have only encountered a single blood of the rare type Rh'Rh'', and this was a blood sample sent to us by Dr L. J. Unger for confirmation. It will be noted that one blood of the rare type Rh₁Rh₂ and 2 bloods giving intermediate reactions were encountered in the present series. The blood of subtype Rh'Rh' from family No 79 mentioned above is the only blood of this rare subtype that we encountered to date, no blood of this subtype occurred in our random series of 645 individuals.

The calculated gene frequencies based on the 645 random bloods, using the formulae given in a previous paper¹ are as follows: $r = 35.9\%$, $R' = 1.2\%$, $R'' = 0.6\%$, $r^0 = 2.5\%$, $R^1 = 44.9\%$ and $R^2 = 16.9\%$.

Therefore, $D = 100 - \Sigma R = -2.0\%$

$PE_D = \pm 0.8\%$

Accordingly, the findings agree satisfactorily with the 6-gene theory. This may seem paradoxical because it is now known that there are more than 6 genes in the Rh

TABLE IV
Summary of Authors' Family Studies to Date

Mating	No of families	No of children of types							Totals
		rh	Rh ₁	Rh ₂	Rh ₁ Rh ₂	rh ₀	Rh'	Rh''	
rh × rh	7	18	0	0	0	0	0	0	18
rh × Rh ₁	82	74	122	0	0	9	0	0	165
rh × Rh ₂	23	11	0	30	0	1	0	0	42
rh × Rh ₁ Rh ₂	32	(1)	30	29	0	0	0	0	60
rh × rh ₀	4	3	0	0	0	4	0	0	7
rh × Rh'	2	1	0	(1)	0	0	1	0	3
rh × Rh''	1	1	0	0	0	0	0	5	6
Rh ₁ × Rh ₁	44	7	86	0	0	4	1	0	98
Rh ₁ × Rh ₂	28	10	20	9	18	0	2	0	59
Rh ₁ × Rh ₁ Rh ₂	35	0	52	10	30	0	0	0	92
Rh ₁ × rh ₀	2	2	0	0	0	0	0	0	2
Rh ₁ × Rh'	7	0	5	0	0	0	2	0	7
Rh ₁ × Rh''	4	1	2	0	3	0	0	0	6
Rh ₂ × Rh ₂	2	1	0	4	0	0	0	0	5
Rh ₂ × Rh ₁ Rh ₂	12	0	6	12	8	0	0	0	26
Rh ₂ × rh ₀	2	0	0	1	0	4	0	0	5
Rh ₂ × Rh'	1	0	0	1	0	0	0	0	1
Rh ₁ Rh ₂ × Rh ₁ Rh ₂	6	0	3	2	4	0	0	0	9
Rh ₁ Rh ₂ × rh ₀	3	0	2	1	0	0	0	0	3
Rh ₁ Rh ₂ × Rh'	2	0	1	1	2	0	0	0	4
rh ₀ × Rh'	1	0	0	0	0	1	1	0	2
Totals	300	90	329	101	65	23	7	5	620

steiner and Wiener of the inheritance of the Rh factor as a simple Mendelian dominant. Similarly, when discussing the genetic relationship of factors Rh' and Rh'' one may say, without danger of ambiguity, that these factors are transmitted by means of a pair of allelic genes *Rh'* and *Rh''* analogous to the genes *M* and *N*. Similarly, if tests are made only with anti-Rh'' and anti-Rh' sera, 2 factors and 3 types would be distinguished which are inherited by a pair of allelic genes *Rh'* and *Rh''*.

Finally, the simplified nomenclature proposed here avoids the introduction of any new symbols or letters, like C, D and E, which are entirely foreign to the scheme of the Rh blood types.

Results Our list of family material is given in Table II.

First, it should be mentioned that there are no contradictions to the laws of inheritance of the A-B-O groups, A₁-A₂ subgroups or M-N types. It is of interest to note that one individual of subgroup A₀ was encountered in family No. 89.

In Table III we have summarized the Rh tests without taking into account the Hr reactions. In a total of 109 families with 173

children there is not a single exception to the theory of 6 allelic Rh genes. When these results are combined with our previously published findings, we have to date a total of 300 families with 620 children tested for the 8 Rh blood types (cf. Table IV). As has already been mentioned, the 2 apparent contradictions to the theory were encountered in our previous studies and both proved to be due to illegitimacy.

In Table V are summarized the families in which Hr tests were carried out in addition to the Rh tests. It will be seen that when either or both parents belonged to type rh, rh₀, Rh₂ or Rh'', none of the children belonged to subtype Rh₁Rh₁ or type Rh'Rh'', on the other hand, when either or both parents belonged to subtype Rh₁Rh₁, none of the children belonged to type rh, rh₀, Rh₂ or Rh''. This confirms the theory that factors Rh' and Rh'' are related to each other as M is to N. In 13 families rh × Rh₁Rh₁ all 24 children belonged to subtype Rh₁rh in conformity with the theoretical requirements,

§ In Table IV, the Rh₁Rh₂ × Rh₁Rh₂ matings from our very first study¹ have been omitted because these appeared to contain some technical errors.

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Mating	No of families	No of children of types (or subtypes)								Totals
		rh	Rh ₁ rh	Rh ₁ Rh ₁	Rh ₂	Rh ₁ Rh ₂	rh ₀	Rh'rh	Rh'Rh'	
rh × rh	2	2	0	0	0	0	0	0	0	2
rh × Rh ₁ rh	10	5	11	0	0	0	1	0	0	17
rh × Rh ₁ Rh ₁	13	0	24	0	0	0	0	0	0	24
rh × Rh ₂	5	1	0	0	6	0	1	0	0	8
rh × Rh ₁ Rh ₂	11	0	6	0	12	0	0	0	0	18
rh × rh ₀	1	0	0	0	0	0	1	0	0	1
Rh ₁ rh × Rh ₁ rh	8	2	7	3	0	0	0	0	0	12
Rh ₁ rh × Rh ₁ Rh ₁	6	0	5	3	0	0	0	0	0	8
Rh ₁ Rh ₁ × Rh ₁ Rh ₁	2	0	0	2	0	0	0	0	0	2
Rh ₁ rh × Rh ₂	3	3	3	0	1	0	0	0	0	7
Rh ₁ rh × Rh ₁ Rh ₂	6	0	0	4	2	4	0	0	0	10
Rh ₁ Rh ₁ × Rh ₁ Rh ₂	2	0	0	1	0	1	0	0	0	2
Rh ₁ rh × Rh'rh	2	0	0	0	0	0	0	1	1	2
Rh ₁ Rh ₁ × Rh'rh	2	0	1	1	0	0	0	0	0	2
Rh ₂ × Rh ₁ Rh ₂	4	0	3	0	2	0	0	0	0	5
Rh ₂ × Rh rh	1	0	0	0	1	0	0	0	0	1
Rh ₁ Rh ₂ × Rh ₁ Rh ₂	2	0	0	2	1	1	0	0	0	4
rh ₀ × Rh ₁ Rh ₂	1	0	1	0	0	0	0	0	0	1
Totals	81	13	61	16	25	6	3	1	1	126

on the other hand, in 10 matings $rh \times Rh_1rh$ with 17 children there were as many as 5 children of type rh and one child of type rh_0 . The most unusual case is family No 79. Here the father belonged to subtype Rh_1rh , the mother to subtype $Rh'rh$, while the daughter belonged to subtype $Rh'Rh'$. As shown in Table I, individuals of subtype Rh_1rh may belong to any one of the 3 possible genotypes, $R'r$, $R'r^0$ or $R'r^0$. In order for the couple in question to have a child of subtype $Rh'Rh'$, it is evident that the father must belong to genotype $R'r^0$.

Another interesting case is family No 45. The mother belonged to type rh, her husband to type Rh_1rh , the older daughter to type rh and the younger daughter to type rh_0 . Obviously the husband could not be the father of both daughters so we concluded that the first child was probably the result of a former marriage. When we called the mother back for a private interview she told us that such was indeed the case and that she had not mentioned it before because she did not want to discuss the matter in the presence of her daughters.

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The calculated gene frequencies based on the 645 random bloods, using the formulae given in a previous paper¹ are as follows: $r = 35.9\%$, $R' = 1.2\%$, $R'' = 0.6\%$, $r^0 = 2.5\%$, $R^1 = 44.9\%$, and $R^2 = 16.9\%$.

Therefore, $D = 100 - \Sigma R = 2.0\%$

$PE_D = \pm 0.8\%$

Accordingly, the findings agree satisfactorily with the 6-gene theory. This may seem paradoxical because it is now known that there are more than 6 genes in the Rh

allelic series²³ The explanation is that the other genes are quite rare The observed value for the frequency of type Rh₁Rh₁ (20.2%) agrees closely with the theoretically calculated value (20.0%) under the theory that factor Hr' is related to factor Rh' as M is related to N

As has been pointed out¹³ the Hr factor considerably increases the chances of exclusion in cases of disputed parentage On Jan 31, 1946, one of us (W) testified in a case before the Court of Special Sessions of New York County, in which the defendant was excluded because he belonged to subtype Rh₁Rh₁ while the child belonged to type Rh₂ This case was tried before a very progressive Bench comprising Judges Nathan Perlman, John B Flood and Irving Ben Cooper

Though this was the first time an Rh-Hr exclusion was presented in a courtroom, the defendant was acquitted, primarily on the basis of the blood test findings

Summary New additions to and further simplifications of the designations of Rh blood types, subtypes and genes are proposed in order to encourage the more general use of the Rh and Hr tests by medical men and geneticists

Investigations on the hereditary transmission of the 8 Rh blood types in 300 families with 620 children yielded results in conformity with the theory of 6 major allelic genes

Studies on the Hr factor in 81 families with 126 children and on a random series of 645 Caucasians in New York City fully support the theory that factors Rh' and Hr' are related to each other genetically and serologically like the agglutinogens M and N

²³ Wiener, A S, *Science* 1945, 100, 595

15329 P

Pathogenesis of Erythroblastosis Fetalis *

ALEXANDER S WIENER

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The present accepted theory of isoimmunization in pregnancy as the cause of congenital hemolytic disease does not explain the manifold clinical manifestations, nor why in the majority of cases no abnormal isoagglutinins can be detected in the maternal serum, nor why in some cases where the maternal serum contains potent agglutinins, the infant is perfectly normal though its red cells contain the specific agglutinin These puzzles have been solved by the discovery^{1,2,3} that individuals sensitized to agglutinogens

such as Rh, Hr, A, B, etc, may form 2 sorts of antibodies, namely, agglutinins (bivalent antibodies) and/or blocking antibodies or glutinins (univalent antibodies) The clinical manifestations depend on the type of isoantibody present in the maternal serum, so according to the present writer's theory erythroblastosis fetalis comprises 2 disease syndromes instead of only one The characteristics of these disease syndromes and the author's proposed terminology are as follows

(1) *Congenital hemolytic disease* This syndrome is caused by the presence in the maternal serum of univalent antibodies specific for the fetal red cells When only small amounts of antibody get into the fetal circulation, the infant is born alive, but develops an anemia which readily responds to proper transfusion therapy When larger

* Aided by a grant from the United Hospital Fund of New York City

¹ Wiener, A S, *Proc Soc Exp Biol and Med*, 1944 56, 173

² Wiener, A S, *J Lab and Clin Med*, 1945, 30, 662

³ Wiener, A S, *J Lab and Clin Med*, 1945, 30, 957

amounts are present in the maternal serum, so that more passes into the fetus, the process progresses further *in utero* until the degree of anemia is such that anoxemia of the capillary walls permits exudation of serum proteins into the tissue spaces. A still-birth results, or a living infant with hydrops, an almost invariably fatal condition. In these "pale" cases the characteristic pathological findings are marked pallor, ascites, anasarca, splenomegaly, hepatomegaly, and microscopically extramedullary islands of hematopoiesis in the liver and spleen and hemosiderosis of the liver and spleen.

(2) *Erythroblastosis proper* Since agglutinins are presumably larger molecules than blocking antibodies, these only rarely traverse the placenta during pregnancy. During labor and delivery, however, increased intrauterine pressure may milk agglutinins into the fetal circulation. In such cases, physical examination of the newborn infant reveals no abnormality at first, but within a few hours a deep jaundice, with little or no anemia supervenes, the infant takes its feedings poorly, becomes stuporous, may have convulsions, coma and death usually supervene. The syndrome is due to the agglutination of the fetal red cells with the formation of agglutination thrombi in the smaller ves-

sel and resulting damage to the liver and brain, in particular. Postmortem examination of such "toxic" cases shows generalized icterus, splenomegaly, and hepatomegaly, microscopically agglutination thrombi in the vessels of the brain are readily demonstrable. Thus, kernicterus is not caused by the jaundice *per se*, but represents an *in vivo* staining reaction of ganglion cells damaged by interference with the circulation. Fortunately, only few infants with this syndrome survive. Those that do are susceptible to the development of signs of a severe neurological disorder, characterized particularly by choreo-athetosis, often associated with mental deficiency, and cirrhosis of the liver.

The new concept of 2 distinct clinical entities instead of one has important implications in the prognosis, treatment and prophylaxis of the 2 disorders. It is now evident that the statement that modern transfusion therapy may only serve to save infants for the worse fate of mental deficiency is misleading, because in the author's experience all of 20 infants saved by such treatment have developed into perfectly normal children. Fortunately, the infants with the syndrome of erythroblastosis who are apt to develop mental deficiency as a sequel rarely survive.

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Antibiotic Activity of Extract of Western Red Cedar Heartwood

CHESTER M. SOUTHAM* (Introduced by B. C. Seegal)

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It is common knowledge that certain woods are highly resistant to decay. One such wood is western red cedar (*Thuja plicata* D. Don) which when extracted in hot water yields a

substance antibiotic to wood-destroying fungi.^{1,2,3} The present paper is a study of the activity of this material against microorganisms pathogenic to man.

The hot water extract of western red cedar

* The author wishes to thank the many people of the Columbia University College of Physicians and Surgeons who by encouragement and ready co-operation have made this study possible. He is especially indebted to Dr. E. T. Engle of the Department of Anatomy.

¹ Schmitz, H., *Idaho Forester*, 1922, 4, 46.

² Sowder, A. M., *Ind. and Eng. Chem.*, 1929, 21, 981.

³ Southam, C. M., and Ehrlich, J., *Phytopath.*, 1942, 33, 517.

TABLE I
Growth of Various Bacteria on Buffered Nutrient Agar Containing Western Red Cedar Extract *

Organism	Concentration of WRC extract in medium		
	57 mg%	115 mg%	162 mg%
<i>Streptococcus hemolyticus</i> (15A)	0	0	0
" " (C203)	0	0	0
" " <i>viridans</i>	+++	++	++
" " <i>fecalis</i>	+++	++	++
<i>Staphylococcus aureus</i> (Oxford)	+++	+	0
" " (B D)	++	++	++
" " <i>albus</i>	+	0	0
<i>Bacillus subtilis</i>	++	++	0
<i>Corynebacterium xerose</i>	0	0	0
<i>Mycobacterium tuberculosis</i> (H37)	++++	++++	+
" " <i>smegmatis</i>	++++	++++	not tested
<i>Klebsiella pneumoniae</i>	++++	++++	++
<i>Enterobacteria typhosa</i>	+++	++	+
<i>Salmonella schottmulleri</i>	+++	++	++
" " <i>paratyphosa</i>	++++	+++	++
<i>Shigella dysenteriae</i>	0	0	0
" " <i>sonnei</i>	0	0	0
" " <i>paradysenteriae</i>	0	0	0
<i>Escherichia coli</i> (communis)	++++	++++	+++
" " (hemolyticus)	++++	+++	+++
<i>Alcaligenes fecalis</i>	0	0	0
<i>Proteus vulgaris</i>	++	++	+
" " (O\19)	++	++	+
<i>Pseudomonas aeruginosa</i>	++++	++++	+++

* ++++ indicates growth equal to that on control plates

+++ " " distinctly but not markedly less than on control plates

++ " " markedly inhibited

+ " " of a few colonies only

0 " " no growth

heartwood (hereafter designated WRC extract) was prepared by covering freshly made cedar sawdust† with distilled water, and simmering for 48 hours. The crude filtrate from this decoction was used in all tests. Concentration is expressed as mg of total solids per 100 cc of solution (mg%). This method is inaccurate because there are probably inert compounds in the extract, and because batches of extract from different trees have shown as much as fourfold differences in antibiotic activity in a given concentration‡. Most figures reported here were obtained with a

single batch of extract made in 1945. In some of the studies with bacteria another less effective, batch of extract was used, but such data have been recorded as those concentrations of the 1945 batch having an equal antibacterial activity.

The media used were prepared by making a stock beef infusion broth or agar twice as concentrated as usual, and containing disodium and monosodium phosphates to give a pH of 7.2 and a concentration of 0.04 molar phosphate. Just before use the desired amount of WRC and enough additional water to double the original volume of the medium was added. Buffering was necessary to eliminate antibiotic activity due solely to the acidity of the WRC extract. As prepared, the reaction of the media varied from pH 7.2 with low concentrations of extract to a maximum acidity of pH 6.6 with the highest concentrations of extract. The solid media were streaked with 24-hour broth cultures

† Wood supplied by B. J. Carney & Co., Spokane, Washington.

‡ WRC extract concentrations have been reported in terms of g of wood represented per 100 cc of extract by Southam and Ehrlich.³ A "10%" concentration, as used in that publication, had approximately the same antibiotic activity as a "100 mg%" concentration in the present study.

TABLE II
Growth of Fungi in Media Containing WRC Extract (Symbols explained in Table I)

Fungus	Culture No	Concentration of WRC extract in media							
		Buffered medium				Unbuffered medium			
		57 mg%	115 mg%	162 mg%	345 mg%	15 mg%	29 mg%	57 mg%	115 mg%
<i>Cryptococcus neoformans</i>	1473	+	0	0	0	+	+	0	0
" "	3	++++	++	+	+	++++	++++	0	0
<i>Candida albicans</i>	21	++++	++	+	0	++++	++++	++++	0
" "	22	++++	++	+	0	++++	++++	++++	0
" "	23	++++	++	+	0	++++	++++	++++	0
" "	24	++++	++	+	0	++++	++++	++	0
<i>Trichophyton gypsum</i>	25	++	++	0	0	++++	++	0	0
" "	26	+	+	0	0	++++	++	0	0
" <i>purpureum</i>	27	++	+	0	0	++++	++	++	0
" "	28	++	+	0	0	++++	++	0	0
" "	29	++	+	0	0	++++	++++	0	0
<i>Epidermophyton floccosum</i>	30	+	+	0	0	++++	++++	+	0
<i>Microsporium felinum</i>	31	++	++	0	0	++++	++	0	0
" <i>audouinii</i>	33	++	+	+	0	++++	++	0	0
" "	34	++	0	0	0	++++	++	+	0
" "	35	+	0	0	0	+	+	0	0

of the test organism. Liquid media were inoculated with 0.1 cc of an undiluted 12-hour broth culture of the test organism. Degree of growth after 24 hours incubation at 37°C on WRC containing media was estimated by comparison with growth on control medium. Growth was recorded as normal (++++), slight stasis (+++), marked stasis (++), very slight growth (+), and no growth (0). *Myc tuberculosis* and *Myc smegmatis* were grown in test tubes on glycerol-agar containing WRC extract and were incubated for 8 weeks, and 4 days, respectively. Santon's synthetic liquid medium containing WRC extract was inoculated with bits of 3-week-old pellicle of *Myc tuberculosis* and incubated 4 weeks.⁵

Degrees of growth of a variety of bacteria on agar media in the 3 concentrations of WRC extract tested are recorded in Table I. It will be seen that there is no relation between staining reaction or morphology of the organisms and their susceptibility to the antibiotic action of WRC extract. There is great variation in susceptibility among different species. Results similar to those recorded were obtained by using liquid medium plus WRC extract.

⁵ Tests using Santon's medium were conducted by C. J. Duer, Department of Bacteriology.

Fungi pathogenic to man were studied by similar technic, with both buffered and unbuffered malt agar medium (2½% desiccated malt extract in 2% agar). The yeast-like fungi (*Cryptococcus* and *Candida*) were streaked in the same manner as bacteria from 4-day maltose broth cultures and the results were read after 4 days. Filamentous fungi were studied by making a single planting 5 mm in diameter in each concentration and comparing the rate of peripheral growth of the mycelium with that on control medium after 7 days. Degree of growth is recorded in the same way as for the bacteria. Incubation was at 37°C.

Results of the fungus growth studies are summarized in Table II. In general the tested fungi were inhibited by the same range of concentrations of WRC extract as the bacteria, when on buffered medium, and by ¼ to ½ these concentrations on unbuffered medium. Fungi causing wood decay are inhibited by similar concentrations.³

The effect of WRC extract on the growth of the Shiga dysentery bacillus was studied by the colony-count (plating-out) technic. Before incubation there were 10,000 viable organisms per cc of medium (buffered nutrient broth plus WRC extract) in all tubes. The control tubes contained 10,000,000 viable

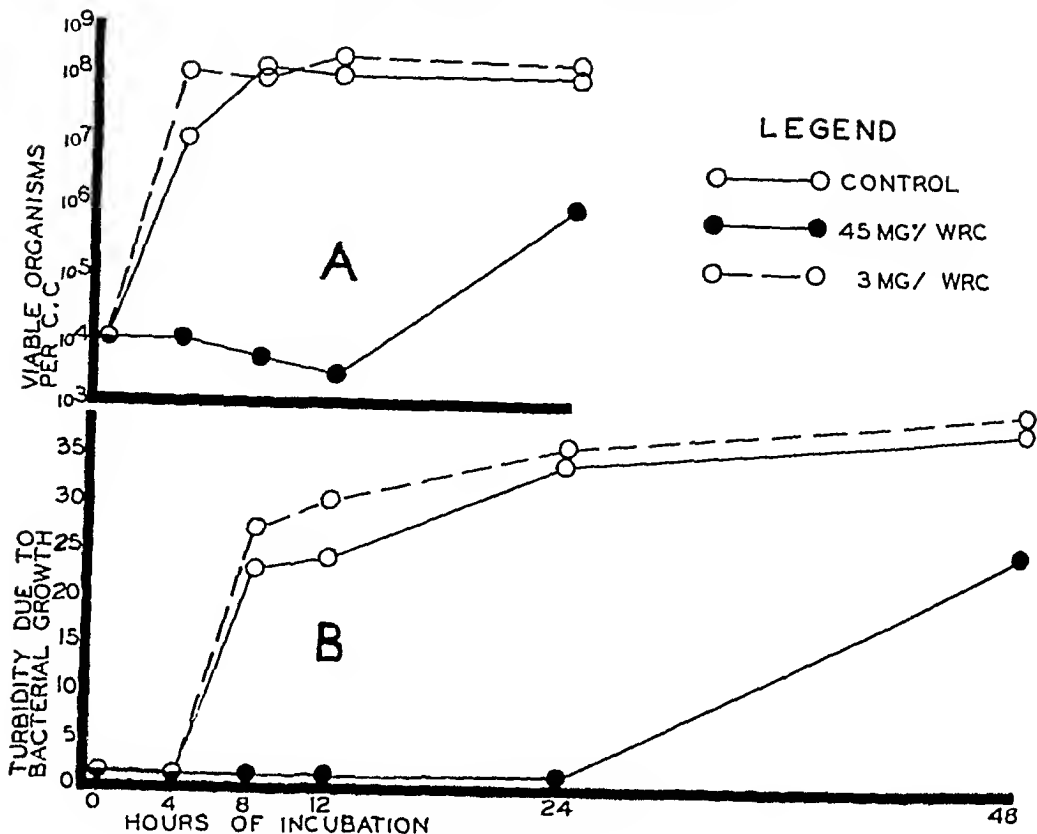


Fig 1
Growth of *Shigella dysenteriae* in buffered nutrient broth containing WRC extract and in control medium (i) Colony count technique, (b) turbidimetric technique

organisms per cc after 8 hours incubation at 37°C. There was no significant change in number of viable organisms in the next 16 hours. In the tubes containing 45 mg% WRC extract there was no increase in viable organisms during the first 12 hours of incubation, but there was an increase to 1,000,000 after 24 hours. These results show that the anti-bacterial activity of WRC extract in the concentration tested is due to stasis, not to death of the organisms (Fig 1A). A second experiment by this same technique confirmed these results.

Growth was also studied in these same tubes by turbidimetric readings taken simultaneously with sampling for the colony-count studies. A Klett-Summerson photo-electric colorimeter was used. A dark red (No 630) filter eliminated variations due to differences in depth of color of the media. Readings

were obtained in units of optical density (log incident light/log transmitted light). Each reading designated in Fig 1B was obtained by subtracting the reading obtained before incubation from that obtained after the indicated period of incubation. These figures therefore represent turbidity due to bacterial growth, not total turbidity of the culture. These results (Fig 1B) also demonstrate that WRC extract is bacteriostatic rather than bactericidal. These results agree with previous findings as to mode of action of WRC.

|| Comparison of the growth curves obtained simultaneously by the 2 techniques (Fig 1A and 1B) shows that the cultures contained nearly the maximum number of living organisms before any turbidity could be detected (i.e. after 4 hours incubation in the controls). Increase in turbidity after this time must be due to accumulation of dead cells.

against wood-destroying fungi³

Microscopic examination of suppressed cultures of several species revealed many elongated rods or enlarged cocci, as has been described previously for organisms exposed to other antibiotics⁴

The mechanism of the bacteriostatic action of WRC extract is unknown. However, it was learned that at room temperature at pH 7.4 WRC extract reacts instantaneously with cysteine in evacuated Thunberg tubes in such manner that the cysteine is rendered unable to reduce methylene blue. This reaction required a minimum of approximately 230 mg of WRC extract solids per milliequivalent weight of cysteine hydrochloride ($\frac{1}{2}$ cc of 1:5000 methylene blue as indicator). WRC extract does not act as a general oxidizing agent for other easily oxidized substances (KI , H_2S , Fe^{++}) so it is probable that the WRC extract does not oxidize cysteine to cystine, but rather reacts directly with the sulfhydryl group. If this be true it is possible that reaction of WRC extract with sulfhydryl compounds is the mechanism, or one of the mechanisms, of its antibiotic action.⁵ The product of reaction of WRC extract with cysteine has no antibiotic activity. WRC extract is also inactivated by human serum and blood. It is not affected by normal urine or by gastric residue.

The dose of WRC extract lethal for animals has not been determined. Mice were maintained for 4 weeks drinking no liquid other than WRC extract (230 mg%), ingesting approximately 230 mg of solids per mouse (11.5 g/K), without toxic symptoms, without weight loss, and without change in volume of liquid consumed. A single subcutaneous injection of 140 mg of WRC extract solids into mice had no ill effects and appeared to cause no discomfort. Intravenous doses of 230 mg (50 cc volume) twice a day for 3 days in a rabbit, making a total of 3 g/K, caused no apparent ill effects, and caused no

grossly detectable pathology. Six mice which were each injected intraperitoneally with 7 mg doses twice a day for 6 days (total of 43 g/K) showed no toxic manifestations. These mice were also drinking WRC extract (230 mg%) throughout this period as their sole source of liquid. Autopsy of 3 of these mice revealed a few scattered discrete white areas up to 2 mm in diameter in their livers. Histological examination revealed that these were fibrosing areas of necrosis containing polymorphonuclear cells as well as macrophages. No other gross pathology could be detected. Kidneys were histologically normal. No other tissues were sectioned. This hepatic pathology may be an effect of the WRC extract. However, the absence of similar pathology in the rabbit, and the discrete character of the lesions, suggests that this pathology may have had some other, possibly infectious, etiology.

Limited trials have failed to reveal any significant antibiotic effect of WRC extract against systemic infection in mice by pneumococcus type III, *Streptococcus hemolyticus* strain C203, *Candida albicans*, or *Cryptococcus hominis*. The possibility of its use against skin infections has not yet been investigated.

The bacterial content of the cecums of normal mice and of mice which had been drinking WRC extract as their only liquid for the preceding week was studied by emulsifying 1 mg of the cecal contents in 10 cc of broth, diluting serially, and growing out in nutrient agar. No significant reduction in number of bacteria was caused by the WRC extract.

Urine from mice and rabbits injected with WRC extract was found to have a greater antibacterial effect than urine from control animals. It seems probable therefore, although the results are not conclusive, that WRC extract is excreted in part in bacteriostatic form by the kidneys.

WRC extract is completely dialyzable through cellophane membrane, but does not diffuse through agar. Boiling to dryness causes little or no loss of antibiotic activity. Heating to 200°C (in sealed vials in boiling benzyl alcohol) momentarily or for 10 min-

³ Waksman, S. A., *Microbial Antagonisms and Antibiotic Substances*, Commonwealth Fund, 1945, 50, 213-215.

⁵ Cavallito, C. J., Briley, J. H., Haskell, T. H., McCormick, J. R., and Warner, W. F., *J. Bact.*, 1945, 50, 61.

utes resulted in loss of 30% of activity. Acidification to pH 1 with HCl, for 48 hours, followed by neutralization with NaOH, caused no detected loss of activity. Alkalinization to pH 12 for one hour, followed by neutralization with HCl, caused no detected loss of activity, but when held at this pH for 48 hours there was roughly 50% loss of activity. At least 10% of the antibiotic activity resides in ether-soluble resinous components of the extract. The active components can be almost completely removed from WRC extract by extraction with benzyl alcohol or by adsorption onto norite, but the active components have not yet been recovered from these reagents.

Summary Water extract of western red cedar heartwood inhibits growth of a wide variety of bacteria and fungi. Inhibition is due to stasis, not to death, of the organisms. Susceptibility of microorganisms is unrelated to Gram-staining reaction or to morphology.

WRC extract does not lose its antibiotic activity when subjected to prolonged boiling, or considerable changes in pH. It is not inactivated by urine or gastric residue, but is inactivated by blood, serum, and cysteine.

Large doses of the extract (4.3 g/K I.P. in mice, 3 g/K I.V. in a rabbit) did not cause death or illness.

No *in vivo* activity against infection has yet been demonstrated.

15331

Immunological Studies of Dengue Fever and Colorado Tick Fever

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(Introduced by MAX LEVINE)

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Ashburn and Craig¹ demonstrated, by human inoculation studies, that dengue fever was caused by a filterable agent and that it was transmitted by mosquitoes. By similar means it has been demonstrated that Colorado Tick fever is caused by a filterable agent,² but it appears that the development of natural cases of the disease is associated with tick bites.³ The Colorado tick fever and the dengue fever syndromes are both characterized by generalized malaise, headache, leucopenia, and a frequent "saddle-back" or di-phasic temperature curve. They are both self-limiting and non-fatal. The outstanding differential feature seems to be the develop-

ment of a skin rash with many cases of dengue fever and no rash with Colorado tick fever, also an association with ticks in the case of Colorado tick fever and with mosquitoes in the case of dengue fever. In view of the similarity of the clinical pictures of these 2 diseases and since they are both associated with arthropods, some opinions have been expressed⁴ that they both may be caused by related agents.

Human Experiments Since animals are not highly susceptible to either of these agents, it was considered advisable to study their immunological relationship to each other by human inoculation experiments.

Two normal male volunteers were selected for subcutaneous inoculation with 1.0 cc of the Colorado Tick fever agent* (Case I-A-B).

¹ Ashburn, P. M., and Craig, C. F., *Philippine J. of Science*, 1907, B, 2, 93.

² Florio, Lloyd, Stewart, M. O., and Mugridge, E. R., *The Etiology of Colorado Tick Fever*, *J. Exp. Med.*, 1946, 83, 1.

³ Topping, Norman H., Cullyford, J. S., and Davis, G. E., *Public Health Rep.*, 1940, 55, 48.

⁴ Rogers, L., and Megaw, J. W. D., *Tropical Medicine*, The Williams and Wilkins Co., 1944, 146.

* We wish to express our sincere appreciation to Dr. E. R. Mugridge, University of Colorado.

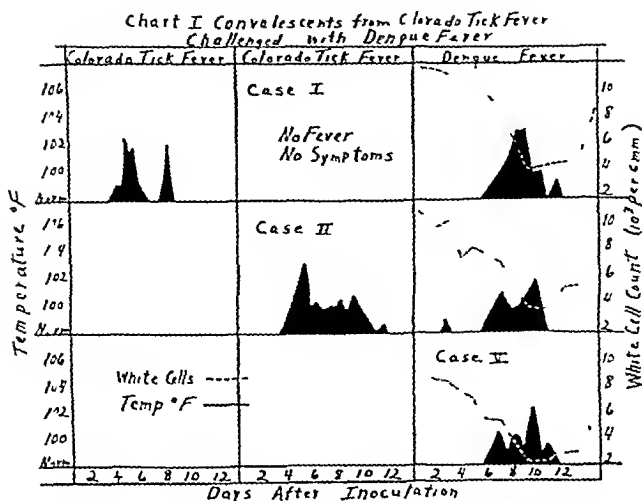


Fig 1

Case I inoculated with Colorado Tick Fever, challenged first with Colorado Tick Fever, then with Dengue Fever. Case II inoculated with Colorado Tick Fever, then challenged with Dengue Fever. Case III inoculated as control for Dengue Fever Virus at time of challenging Cases I and II.

and Case II-APR.) As noted in Fig 1, Case I had manifested experimentally induced Colorado tick fever and was immune when challenged 5 months later with the same strain of the disease agent. The control inoculation (Case II) developed the disease after 4 days and remained ill for 8 days. The inoculum had been collected on the first day of fever from an experimentally induced case of Colorado tick fever and had thereafter been stored for 6 months in the dry ice chamber. Blood serum was collected from Case II on the first day of fever and was stored in the dry ice chamber for future use.

Twenty-seven days after defervescence, the 2 Colorado tick fever convalescent patients and one normal control were inoculated intradermally with 0.4 cc of dengue fever infected serum. The inoculum was collected from Case IV which is described below. All 3 of the volunteers developed a syndrome which was considered compatible with the clinical diagnosis of dengue fever: fever, leucopenia, generalized malaise and rash.

Two normal male volunteers (Case III-CW and Case IV-ET, Fig 2) were inoculated intradermally with 0.4 cc of dengue

fever infected serum (second human passage).⁶ Six days after this inoculation the patients developed symptoms of the disease and the illness persisted for 6 days. Blood serum was collected from these patients during the first day of illness and was stored in the dry ice chamber for future use. Twenty-seven days after defervescence the 2 dengue fever convalescent patients and one normal control were inoculated subcutaneously with 1.0 cc of Colorado tick fever infected serum. This inoculum had been collected from Case II described above. Three and 4 days after the inoculation all of the challenged individuals and the control developed the syndrome of Colorado tick fever (Fig 2): fever, leucopenia, generalized malaise, but no rash.

This reciprocal challenge experiment, therefore, failed to demonstrate any relationship between the dengue fever and the Colorado tick fever strains which were employed.

Discussion There are several diseases reported in the literature, all of which are characterized by fever, by generalized malaise, by leucopenia and by being associated with arthropod infestation. Speculations have been

⁶ The dengue fever agent was kindly supplied by Dr. A. B. Sabin, Children's Hospital Foundation, the University of Cincinnati.

⁶ The dengue fever agent was kindly supplied by Dr. A. B. Sabin, Children's Hospital Foundation, the University of Cincinnati.

Chart II Convalescents from Dengue Fever
Challenged with Colorado Tick Fever

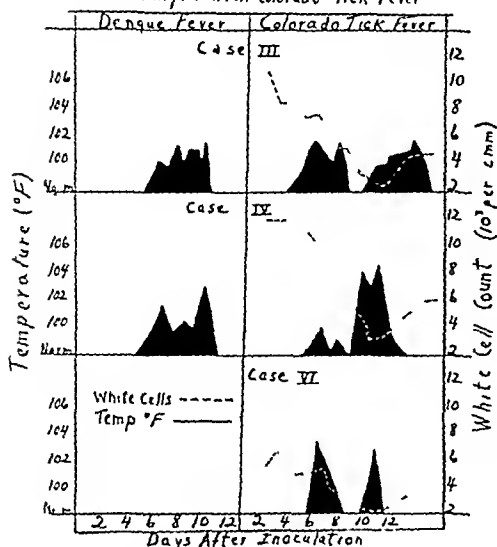


Fig. 2

Cases II and IV inoculated with Dengue Fever then challenged with Colorado Tick Fever. Case VI inoculated with Colorado Tick Fever for control.

proposed concerning their possible similarity in etiology. To great extent, experimental results have served to delineate their relationship to each other. Sandfly fever and dengue fever appear to be caused by immunologically distinct agents.⁵ Bullis fever

⁵ Sabin, A. B., Philip, C. B., and Paul, J. R.,

and Colorado tick fever, while both associated with tick bites, appear to be of different etiology,⁶ furthermore, Bullis fever and dengue fever have not thus far demonstrated any immunological relationship.⁷

Different strains of the same disease may vary somewhat immunologically. It is possible that the relationship of dengue fever and Colorado tick fever with strains other than those employed herein, may not coincide with the results reported. It appears, from our results, however, that there is no immunological relationship between the agents of Colorado tick fever and of dengue fever (strain).

Summary On the basis of the experimental data presented, there does not appear to be any immunological relationship between dengue fever and Colorado tick fever.[†]

J. Am. Med. Assn., 1944, 125

⁶ Pollard, M., Livesay, H. R., Wilson, D. J., and Woodland, J. C., Experimental Studies with Bullis Fever, *Am. J. Trop. Med.*, 1946, in press.

⁷ In press.

[†] A recent article by Florio, Hummon, Laurent, and Stewart appeared in the *Journal of Experimental Medicine*, 1946, 4, 83, in which essentially the same results were obtained as is reported in this paper.

Grateful acknowledgment is made to Major Choate Matthews, M. C., and Captain Sidney Cohen, M. C., for assistance with the clinical observations.

15332

A Hemorrhagic Syndrome Induced by Derivatives of 3-Hydroxy-1,4-Naphthoquinone.*

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(Introduced by L. H. Schmidt)

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During the routine examination of the short-term chronic toxicities of various anti-malarial drugs for the rat, it was noted that certain derivatives of 3-hydroxy-1,4-naphthoquinone produced a fatal hemorrhagic syndrome associated with a profound hypopro-

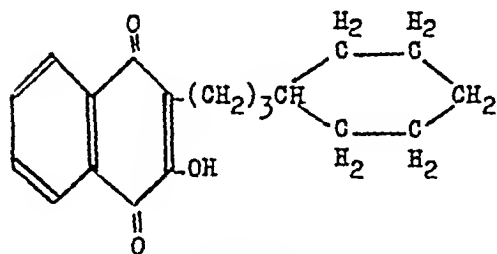
thrombinemia. The production of such a syndrome by drugs related closely in structure to the antihemorrhagic K vitamins, the rapidity with which this syndrome developed and its severity were all items of interest. Especially significant, however, was the find-

* The work described in this paper was done under a contract recommended by the Committee on Medical Research between the Office of Sci-

entific Research and Development and the Institute of Medical Research, The Christ Hospital, Cincinnati, Ohio.

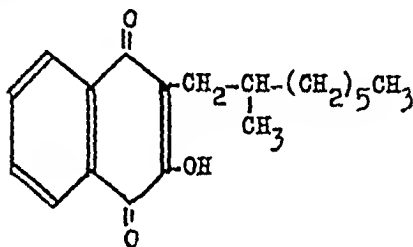
ing that production of hemorrhagic lesions and hypoprothrombinemia by at least one of the above naphthoquinones was blocked by simultaneous administration of vitamin K₁. A description of these observations is presented here.

Experimental The compounds studied were the following



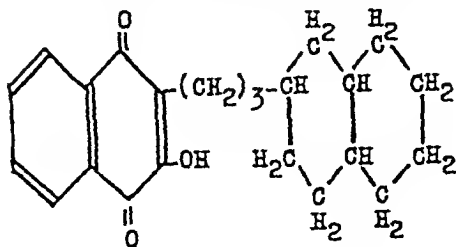
SN 5090†

2 (3 cyclohexyl propyl) 3 hydroxy 1,4 naphthoquinone



SN 5949†

2 (2 methyloctyl) 3 hydroxy 1,4 naphthoquinone



SN 8557†

2 [3 (decahydro 2 naphthyl)propyl] 3 hydroxy 1,4 naphthoquinone (Trans mixture)

The toxicities of these substances were studied in the young rat in accordance with the standardized technics employed with other

antimalarial drugs. Since these procedures will be described in detail elsewhere† only the most important features will be mentioned here. The drugs, suspended either in water or in olive oil, were administered at various dose levels to groups of young rats for periods of 8 to 11 days. Treatments were given once daily via stomach tube. Control rats received similar quantities of water or olive oil without drug at the same intervals. The weights of the animals were obtained daily. In many instances, prothrombin times were measured. Samples of whole blood obtained by cardiac puncture were used for this purpose. Determinations were carried out according to the method of Kato¹ using Russell viper venom² as the source of thromboplastin.

Twenty-eight-day-old male rats, Sprague-Dawley strain, were used throughout the study. The weights of these animals at the start of an experiment averaged 55 g. Throughout the treatment period the rats were fed Purina Dog Chow Checkers and water ad libitum.

A brief summary of the pertinent results of the study is given in Table I. The data presented there show that at certain dose levels each of the 3 compounds produced a hemorrhagic syndrome in the young rat. The first evidence of this syndrome usually appeared after the third or fourth day of treatment. At this time the ears, feet and tails showed unusual pallor and in some cases there was slow bleeding from ear puncture wounds made to identify the animals. Body temperatures were reduced and the rats were weak and inactive. Twenty-four to 72 hours later the animals were usually moribund. Gross extravasations of blood into the subcutaneous tissues of the legs, neck and head, and about the testicles were invariably present. Blood samples obtained from the heart without anticoagulant either failed to clot within 24 hours or clotting was greatly delayed. The hematocrits on such samples

† The numbers cited here are those assigned to the compounds by the Survey of Antimalarial Drugs, Baltimore, Maryland. The drugs were prepared by Dr. Louis F. Fieser, Harvard University, and the Abbott Laboratories, to whom we are greatly indebted.

‡ *Survey of Antimalarial Drugs, 1941-1945*, to be published in July, 1946.

¹ Kato, K., *Am J Clin Path*, 1940, **10**, 147.

² Page, R. C., and Russell, H. K., *J Lab and Clin Med*, 1941, **26**, 1366.

TABLE I
Observations on the Toxicities of Derivatives of 3-Hydroxy-1,4-Naphthoquinone

Daily dose mg/kg	No of rats treated	Deaths due to treatment	Wt change in % controls*	Effects of treatment
SN 5090 — administered in water				
400	6	0	130	} No manifestation of toxicity during 8 days treatment Hemorrhagic syndrome appeared in 3 rats on 5th and 6th days
800	6	0	130	
1600	6	1	75	
SN 5090 — administered in olive oil				
800	4	1	12	} Greatly prolonged prothrombin times in 3 rats surviving 11 days treatment Fatal hemorrhagic syndrome developed in all 5 animals, mean survival time 5 days
1200	3	3	—	
1600	2	2	—	
SN 5949 — administered in water				
100	6	0	140	} No manifestation of toxicity in treatment period of 8 days Hemorrhagic syndrome appeared in 2 rats in 5 days, mean survival time 5 days
200	6	0	85	
400	6	2	75	
SN 5949 — administered in olive oil				
100	5	0	92	} No manifestation of toxicity during 11 days treatment Fatal hemorrhagic syndrome developed in all 5 rats, mean survival time 4 days
200	5	0	84	
400	5	5	—	
SN 8557 — administered in water				
100	6	0	93	} No manifestation of toxicity during 11 days treatment Fatal hemorrhagic syndrome in 4 animals, mean survival time 5 days Fatal hemorrhagic syndrome in all 6 rats, mean survival time 6 days
200	6	4	—	
400	6	6	—	
SN 8557 — administered in olive oil				
100	6	0	57	} No manifestation of toxicity in treatment period of 11 days Fatal hemorrhagic syndrome in 5 animals, mean survival time 5 days Fatal hemorrhagic syndrome developed in all 6 rats, mean survival time 6 days
200	6	5	—	
400	6	6	—	

* The figures in this column were obtained as follows

$$\frac{\text{G wt change of treated rats}}{\text{G wt change of control rats}} \times 100$$

were extremely low—in some instances only 15%. Necropsies presented a fairly uniform gross picture—bloodless appearance of such organs as liver, kidney, spleen and pancreas, gross hemorrhage into the abdominal cavity and the upper portions of the gastro-intestinal tract without distinct ulceration, considerable partially digested blood in the lower parts of the digestive tract.

The first appearance of this syndrome suggested the presence of a prothrombin de-

ciency. Consequently prothrombin times were measured routinely whenever blood samples could be obtained. The results of these determinations may be summarized briefly. In most instances prothrombin times were normal in animals which showed no bleeding tendency, in a very few cases, however, there were slight but significant elevations of the prothrombin times. In contrast to this, rats presenting the hemorrhagic syndrome had greatly prolonged prothrombin times, varying

from 300 seconds to in excess of 24 hours. With the method employed here, normal rats have a prothrombin time of 18 ± 3 seconds.

It should be pointed out that there were some quantitative differences in the toxicities of the 3 drugs employed here. SN 5090 was distinctly less toxic than SN 5949 or SN 8557. SN 5090 appeared to be considerably more toxic when administered as a suspension in olive oil than when suspended in water. The menstruum had little effect on the toxicities of SN 5949 and SN 8557.

In both the experiments described above and in collateral studies, data have been sought which would explain the observed hypoprothrombinemia. One of the first possibilities considered was that the hypoprothrombinemia resulted from hepatic injury. This explanation does not seem likely, for in only one of the rats was there distinct microscopic evidence of liver pathology. In the liver of this animal there was marked fatty degeneration and areas of coagulation necrosis about the central vein. It is noteworthy that more severe changes than these have been observed in hundreds of rats receiving other potential antimalarial drugs, but in none of these animals was there a hemorrhagic syndrome or evidence of hypoprothrombinemia.

The second explanation which suggested itself was that, as in the case of the indandiones,³ another group of hemorrhagic agents, the naphthoquinones effected a direct inactivation of prothrombin. To determine this point, prothrombin times were measured before and at various intervals up to 8 hours after normal rat plasma had been saturated with either SN 5090 or SN 5949. Without exception prothrombin times were the same in plasmas saturated with these drugs as in the control plasmas. This result argues against direct inactivation of prothrombin by the naphthoquinones.

Another possible explanation was that the hypoprothrombinemia resulted from a vitamin K deficiency induced by interference with bacterial synthesis of these vitamins in the intestine—an effect not unlike that induced

by the sulfonamides.⁴ *In vitro* experiments have shown, however, that neither SN 5090 nor SN 5949 possesses significant bacteriostatic or bactericidal activities against *Escherichia coli*,⁵ the intestinal organism regarded as the principal bacterial source of the K vitamins.⁶ Furthermore, the hypoprothrombinemia associated with sulfonamide therapy develops only after weeks or months of treatment, the hypoprothrombinemia described here appears in a matter of a few days. Thus, at the present moment there seems little to support the explanation suggested above.

Another explanation was that drugs such as SN 5090 competed with the K vitamins in the formation of prothrombin—the relationship being not unlike that which appears to exist between the sulfonamides and p-aminobenzoic acid, or between pantooyltaurine and pantothenic acid. The explanation suggested itself, not only because of the structural similarities of substances with vitamin K activity and the above hemorrhagic agents, but also because of Richardson's finding,⁷ that the activities of the above naphthoquinones against infections with *Plasmodium lophurac* were at least partially blocked by 2-methyl-1,4-naphthoquinone, sometimes called synthetic vitamin K.

To test the above postulate, an experiment was set up to determine whether the toxic reactions produced by SN 5090 could be blocked by simultaneous administration of either vitamin K₁ (2-methyl-3-phytyl-1,4-naphthoquinone) or 2-methyl-1,4-naphthoquinone. The results of this experiment, summarized in Table II, show rather dramatically that the simultaneous administration of vitamin K₁ at a daily dosage level of 10 mg per kg blocked completely the appearance of the hemorrhagic syndrome and hypoprothrombinemia in rats receiving SN 5090. Furthermore, vitamin K₁ blocked the

⁴ Black, S., Overman, R. S., Elvehjem, C. A., and Link, K. P., *J. Biol. Chem.*, 1942, **145**, 137.

⁵ Sesler, C. L., and Schmidt, L. H., unpublished observations.

⁶ Dam, H., *Advances in Enzymology*, 1942, **2**, 285.

⁷ Personal communication from L. F. Fieser.

³ Kabat, H., Stohlman, E. F., and Smith, M. I., *J. Pharm. and Exp. Therap.*, 1944, **80**, 160.

TABLE II
The Effects of 2 Methyl 1,4 Naphthoquinone and Vitamin K₁ on the Toxicity of SN 5090 *

Rat No	Days of treatment	Wt, g		Prothrombin time† sec	Remarks
		Initial	Final		
Rats received 800 mg SN 5090 per kg body weight, daily					
1	4	57	53	240	Moribund, sacrificed on 5th day, fatal hemorrhagic lesions
2	4	56	54	180	
3	7	54	57	—	Died 7th day, fatal hemorrhagic lesions
4	7	58	70	—	
5	8	55	67	180	Moribund, sacrificed 8th day, fatal hemorrhagic lesions
6	8	57	70	240	
Rats received 800 mg SN 5090 and 10 mg 2 methyl 1,4 naphthoquinone per kg body weight, daily					
7	12	56	70	150	Sacrificed end of treatment, moderately severe hemorrhagic lesions
8	12	64	88	150	
9	12	57	88	95	
10	12	60	101	95	
11	12	50	90	50	Sacrificed end of treatment, nothing remarkable
12	12	59	100	40	
Rats received 800 mg SN 5090 and 10 mg vitamin K ₁ per kg body weight, daily					
13	12	51	93	20	Sacrificed end of treatment, nothing remarkable
14	12	60	117	19	
15	12	56	108	17	
16	12	55	96	15	
17	12	58	100	15	
18	12	65	112	15	

* SN 5090 was suspended in olive oil, the vitamins were dissolved in olive oil

† Prothrombin times were obtained when animals were sacrificed, either moribund or at end of experiment

growth inhibiting effects of SN 5090, animals receiving both of these naphthoquinones (rats 13-18) gained weight essentially as well as olive oil controls. Although methyl-naphthoquinone was much less effective in counteracting the toxicity of SN 5090 than vitamin K₁, it did delay the appearance of the hypoprothrombinemia significantly, retarded the development of hemorrhagic lesions and partially blocked the growth inhibiting effects of SN 5090. These data would seem to support the postulate that naphthoquinones like SN 5090 produce a hypoprothrombinemia by competing with the K vitamins in processes essential to prothrombin formation.

Discussion The results of the present study have shown that 2-substituted-3-hydroxy-1,4-naphthoquinones, such as SN 5090, produce a hemorrhagic syndrome when administered to the rat in large doses. A rational explanation for this hemorrhagic activity has been offered, namely, that these

quinones function as antimetabolites in whatever processes the K vitamins serve in prothrombin formation. The observation that vitamin K₁ and to a lesser extent methyl-naphthoquinone antagonize the hemorrhagic and other toxic properties of SN 5090 provides good support for this explanation.

A number of other 3-hydroxy-1,4-naphthoquinone derivatives have been reported in the literature. It is significant that at least one of these derivatives, namely, 2,2'-methylene-bis-3-hydroxy-1,4-naphthoquinone, possessed hemorrhagic activity. This compound produced a significant reduction in plasma prothrombin content when administered to rabbits in doses of 20-40 mg⁸. The other 2-substituted-3-hydroxy-naphthoquinones were studied only for vitamin K activity in the chick at low dosage levels of not more than 1 mg⁹. Perhaps some of these compounds

⁸ Meunier, P., Mentzer, G., Buu Hoi, and Cugniant, P., *Bull soc chim biol*, 1943, **25**, 384

⁹ Ahlquist, H. J., and Klose, A. A., *J. Am*

would have displayed hemorrhagic activity had they been tested in rats or rabbits in larger doses. Conversely, the 2-substituted-3-hydroxy-naphthoquinones used in the present study might display antihemorrhagic activities if assayed at low doses in the vitamin K-deficient chick.

Summary 1 A fatal hemorrhagic syndrome accompanied by marked hypoprothrom-

Chem Soc, 1939 **61**, 1924, 2557 Almquist H J, and Klose, A A, *Proc Soc Exp Biol and Med*, 1940, **45**, 55, Fieser, L F, Bowen D M, Campbell W P, Fry E M, and Gates M D Jr, *J Am Chem Soc*, 1939, **61**, 1926, Fieser L F, Campbell W P, and Fry, E M, *J Am Chem Soc*, 1939, **61**, 2206, Fieser, L F, Tishler, M, Simpson, W L, and Woodford, S, *J Biol Chem*, 1941, **137**, 659, Fieser, L F, and Gates, M D, Jr *J Am Chem Soc*, 1941, **63** 2948, Fernholz, E, and Ansbacher, S *Science*, 1939 **90**, 215

binemia was produced in rats by the administration of 3 2-substituted-3-hydroxy-1,4-naphthoquinones

2 These compounds produced only minimal pathologic changes in the liver, caused no measurable destruction of prothrombin *in vitro*, and possessed only slight bacteriostatic activity against *Escherichia coli*, the principal source of vitamin K in the gut

3 The hemorrhagic and toxic properties of at least one of the compounds studied, SN 5090, were antagonized partially by 2-methyl-1,4-naphthoquinone and completely by vitamin K₁. On the basis of this finding it was postulated that these 2-substituted-3-hydroxy-1 4-naphthoquinones, such as SN 5090, compete with the K vitamins in processes essential to prothrombin formation and by displacing these vitamins produce the hemorrhagic syndrome described here

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Chemotherapy of Experimental Leishmaniasis *

H B VAN DYKE AND ALFRED GELLHORN

From the Department of Pharmacology, College of Physicians and Surgeons, Columbia University, New York City

The work here reported was an experimental chemotherapeutic investigation of a variety of organic compounds, both metallic and non-metallic, to determine whether any offered a new approach to the treatment of visceral leishmaniasis. None as suitable as available drugs was discovered. This report will describe the method of evaluating drugs of unknown potency and summarize tests of 188 compounds.

Materials and Methods Few details of the investigative technics of various authors have been published and, in most cases, these technics require much time and have not been well standardized. (A recently-described, ac-

curate but time-consuming method is that of Goodwin)¹ A routine "screening" method should be reliable, rapid and convenient. The method to be described meets these qualifications. However had a potent drug of low toxicity been discovered, the method of comparison with drugs already available would have had to be much more elaborate.

In all experiments the Syrian hamster, *Cricetus auratus*, was used. It was clearly desirable that the dosage of a compound of unknown value be as high as infected hamsters could tolerate. Usually a satisfactory dose could be determined from the estimated murine LD50 following a single intraperitoneal injection of either an aqueous solution of a compound, or, if the compound was insoluble in water, a suspension in 2%

* The work described in this paper was done under a contract, recommended by the Committee on Medical Research, between the Office of Scientific Research and Development and Columbia University.

¹ Goodwin, L G *Trans Roy Soc Trop Med Hyg*, 1945, **39** 133

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⁹ Almqvist, H. J., and Klose, A. A., *J Am*

muth, copper, gold or mercury had no effect on the disease. None of 144 aliphatic, carbocyclic and heterocyclic non-metallic compounds was found to possess important anti-leishmanial effects. Only 4 of these 144 compounds, administered in large doses in terms of toxicity, had any effect, this effect was clearly inferior to that of Stibantoin on

leishmaniasis in hamsters

Summary A method for the routine evaluation of drugs for the therapy of visceral leishmaniasis in the hamster is described. No drug superior to Stibantoin, Neostam or Neostibosan was discovered in tests of 27 other antimonials or of 158 additional metallic and non-metallic organic compounds.

15334

Effects of Inflammation (Turpentine Abscess) on Iron Absorption

P F HAHN, W F BALE, AND G H WHIPPLE

*From the Departments of Pathology and Radiology, University of Rochester,
School of Medicine and Dentistry*

These experiments with radioactive iron are concerned with iron absorption from the intestinal tract as influenced by infection. For many years pathologists have used the turpentine sterile abscess as a means of studying the response of experimental animals to a simple inflammatory reaction. Several dogs were accordingly studied in order to determine how such an inflammation might influence the uptake of labelled iron in single doses. It has been shown that the individual response to iron therapy is quite variable and necessitates control studies in the same subject.¹ The resulting data are summarized in Table I.

Experimental Data Dog 40-149, an adult female mongrel fox terrier, had been maintained by periodic bleeding at an anemic level of blood hemoglobin for about 2½ years. The diet consisted of hospital table scraps. One ml of turpentine was injected in the axilla and the next day a test dose of 10 mg of iron labelled by radioactivity was given by mouth. Subsequent blood samples over a 2-week period showed an uptake of 4.1% of the dose. Control feedings of iron before and after the abscess period showed an average utilization of 29.5% of the dose. The methods used in determina-

tions of labelled iron utilization have been previously described.^{2,3}

Dog 41-164, an adult mongrel female terrier, had been kept anemic by repeated bleeding for a period of one year. The diet consisted of hospital table scraps. One ml of turpentine was injected subcutaneously in the left axilla and 2 days later a test dose of 38 mg of labelled iron as ferric ammonium citrate was given by gavage. Four % of the test dose was utilized in hemoglobin formation. A control feeding of the same amount of iron a month later showed an uptake of 17.6% of the test dose.

Dog 39-196, an adult female mongrel spaniel, had been maintained at a low level of hemoglobin by bleeding over a period of 2½ years, and splenectomized 14 months previous to this experiment. The diet was low in iron, and consisted mainly of white bread, salmon, and dried milk powder. One ml of turpentine was injected subcutaneously in the left axilla. Two days later a single dose of 38 mg of labelled iron was administered as ferric ammonium citrate by gavage. After 9 days 5.3% had been utilized for hemoglobin formation. In an attempted control experiment carried out a month later

¹ Hahn, P F, Jones, Edgar, Lowe, R C, Meneely, G R, and Percock, Wendell, *Am J Physiol* 1945 143 191

² Hahn, P F, Ross, J F, Balfour, W M, Bale, W F, and Whipple, G H, *J Exp Med*, 1942, 75, 221

³ Hahn, P F, *Am J Physiol*, 1944, 141, 363

gum acacia. Ten per cent of the estimated murine LD50 was administered once daily intraperitoneally for 6 days to hamsters under treatment by the method outlined below. In some instances, serious toxic effects as judged by a fall in weight still occurred and adjustment of the daily dose to a lower level had to be instituted. The routinely used standard drug, the diethylamino ethanol of sodium antimony gluconate ("Stibanose" or "Solustibosan") had a very low toxicity represented by an LD50 and standard deviation, after a single intraperitoneal injection into mice, of 3550 ± 925 mg per kg. In contrast to this quinquevalent antimonial, a similar estimate of the LD50 and standard deviation of the tervalent antimonial, tartar emetic, was 60 ± 16 mg per kg.

Standardization of the method of testing routinely the chemotherapeutic action of drugs in experimental leishmaniasis required much time. Preliminary experiments with a strain of *Leishmania infantum*, kindly furnished by Dr S. Adler of the Hebrew University in Jerusalem, produced infections after intrasplenic inoculation but was not satisfactory for routine use because of the difficulty of standardizing the infection. The same objection was found to hold when intratesticular inoculations were made with several strains of *Leishmania*. By using the Khartoum strain of *L. donovani*, which was obtained through Dr Henry Meleney, and by making a rigidly controlled intraperitoneal inoculation of splenic emulsion, well standardized leishmaniasis in groups of hamsters could be produced.

In routine experiments, one or more groups of 100 hamsters about 4 weeks old received inoculation from a single suspension of ground hamster spleen which was heavily parasitized with the Khartoum strain of *L. donovani*. Each animal was given an intraperitoneal injection of uniformly mixed suspension equivalent to 20 mg of spleen. Careful precautions were taken to avoid bacterial contamination. After 4 weeks there was usually a patent infection which was uniform for the group as judged by liver smears obtained by puncture with a 22 gauge needle fitted to a syringe. Each group of

100 was subdivided at random into groups of 5 hamsters. Of these sub-groups, one served as an untreated control, a second received the standard drug, Stibanose, in a daily dose of 500 mg per kg, and the remaining 18 sub-groups received compounds of unknown value. All solutions or suspensions of compounds were injected intraperitoneally once daily for 6 days and a second liver puncture was made on the eighth day—2 days after the last injection. At this time, usually all the hamsters receiving Stibanose were free from leishmaniasis as judged by liver smears, however, in an occasional group, liver smears were still positive in 1 or 2 of the 5 animals. Two weeks later animals treated by Stibanose rarely had positive liver smears whereas cultures of liver in NNN medium were positive in about 20%. The parasitization of control hamsters or of others receiving inactive compounds progressed rapidly until death owing to renal amyloidosis occurred.² Remarkably consistent evaluation of liver smears was obtained "blindly" by independent observers. Results could easily be classified as (1) no chemotherapeutic effect, (2) a chemotherapeutic effect less than that of Stibanose, and (3) a chemotherapeutic effect equal to that of Stibanose. Rarely, a difference between categories (1) and (2) could not be decided until further experiments had been completed. Additional experiments were performed with any drug falling in category (2) or (3).

Results. Tables summarizing data on all compounds not restricted with regard to publication can be secured from the authors. The greatest proportion of active drugs, as might be expected, was found among the 30 antimonials. Only 2 of the 16 tervalent antimony derivatives were active whereas 8 of the 14 quinquevalent antimonials were beneficial in experimental leishmaniasis. No new drug of these groups was as effective as known drugs such as Stibanose, Neostam, and Neostibosan. Fourteen other organometallic compounds containing arsenic, bis-

² Gellhorn, A., and Dyke, H. B., Pyles, W. J., and Tupper, N. A., *Proc. Soc. Exp. Biol. and Med.*, 1946, **61**, 25.

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TABLE I
Iron Absorption by Dogs with Subcutaneous Turpentine Abscesses and During Control Periods

Dog	Date 1943	Hct %	Dose mg	Form	% absorption	Experiment type
40 149	6/17	13.4	9	FeCl ₂	41.0	Control
"	7/20	23.0	10	"	4.1	Turpentine 1 day previous
"	9/13	18.3	10	"	18.1	Control
41 164	1/9	26.9	38	FAC	4.1	Turpentine 2 days previous
"	2/8	28.6	34	"	17.6	Control
37 196	1/9	22.4	38	"	5.3	Turpentine 2 days previous
"	2/8	23.4	34	"	6.9	Control

(This animal had been splenectomized and was therefore liable to *Bartonella* infection. That this infection was present was later indicated by reaction to Mapharsen.)

Balance sheet on 1/9/43 feeding of dog 37 196

1/19 Hct 35.2% estimated CV = 265
2/3 " 28.7

Total circulating R₁Fe = 4720 c/m
" " " = 635

Radioactivity lost to circulation = 4085

Radioactivity found in 1900 ml blood removed in period 1/19 to 2/3 = 4045

This balance indicates that there was no additional absorbed and stored radio iron to be used under the stimulation of blood loss

6.9% of a similar test dose was utilized

This dog subsequently showed a low rate of hemoglobin production on an adequate diet, a result consistent with an infection with *Bartonella canis*, a common finding on splenectomized dogs. This probability was further supported by a much more rapid rise in hemoglobin after administering 18 mg of Mapharsen by vein.

Balance sheet data in Table I show that on subsequent bleeding there was a quantitative accounting for the labelled iron in circulation, but no further mobilization of labelled iron from the tissues under the stress of the resulting anemia. This indicates that infection had prevented the absorption of iron.

Discussion It seems apparent from these 3 experiments that inflammation accompanying the presence of turpentine in the sub-

cutaneous tissues markedly reduces the ability of the dog to absorb iron from the gastrointestinal tract. These findings are of interest because of the frequent occurrence of anemias, often refractory to treatment, associated with infections and inflammatory disease. That the utilization of iron already in the body of dogs may be hindered by infection has also been reported by Robschert-Robbins and Whipple,⁴ and recently by Gibson and Finch (personal communication) in studies on human subjects using radioactive iron.

Summary The radioactive isotope of iron shows that iron absorption from the gastrointestinal tract of the dog is impaired by the presence of a sterile abscess in subcutaneous tissue.

⁴ Robschert-Robbins, F. S., and Whipple, G. H., *J. Exp. Med.*, 1936, **63**, 767.

Electrocardiographic Changes in Hemorrhage and Ischemic Compression Shock*

JOSE MANRIQUE IZQUIETA,[†] AND BERNARD PASTERNAK
(Introduced by Carl J Wiggers)

From the Department of Physiology, Western Reserve University Medical School,
Cleveland, Ohio

Prolonged and severe posthemorrhagic hypotension, as in standardized procedures described in various communications from this laboratory,^{1,2} produces an irreversible state. This is evidenced by the fact that reinfusion of all withdrawn blood only temporarily restores normal blood pressures, within intervals of $\frac{1}{2}$ to 4 hours arterial pressures progressively decline and the animal dies. Evidence has been presented^{3,4,5} that impairment of the myocardium is a factor in this unfavorable response to blood transfusion. If this is true, it is reasonable to expect that a methodical series of electrocardiograms taken during the course of such experiments might manifest some changes.

Ischemic compression shock as produced by Green *et al*⁶ likewise produces progressive circulatory failure which is benefited only temporarily by transfusion of blood or artificial blood substitutes. The changes in effective venous pressures and the reactions of the myocardium to equivalent venous pressures have not been reported.

Methods Dogs, 10-17 kilos in weight, were

anesthetized with morphine (ca 3 mg) and sodium barbital (ca 200 mg/kilo). Standardized hemorrhagic shock was produced in 9 dogs as in previously reported experiments. The animals were bled until mean arterial pressure fell to 50 mm and this pressure was approximately maintained for 90 minutes. At the end of this period pressure was reduced to and maintained at 30 mm for 45 minutes. All withdrawn heparinized blood was then reinfused and the onset of circulatory failure awaited.

Ischemic compression shock was produced in 9 dogs by the method described by Green *et al*⁶. Both hind legs were wrapped tightly from ankle to groin with stretched rubber tubing. This generally resulted in hypertension and dyspnea. After 6 hours the tube wrapping was removed. The arterial pressure fell rapidly to slightly subnormal levels and thereafter progressively declined to 50 or 40 mm Hg in 2 to 6 hours.

In both types of experiments complete series of electrocardiographic leads were taken at frequent intervals. Each series consisted of the 3 standard leads and 4 or 5 precordial leads using the central terminal of Wilson. In some experiments 3 "augmented leads" after the method of Goldberger were taken. Electrodes covered with a layer of cotton saturated with normal saline solutions were placed under the skin and left in place throughout the experiments. Mean blood pressure was recorded continuously from a femoral or carotid artery.

Results in Hemorrhagic Shock With the exception of one dog, all showed normal electrocardiograms during the control period, but revealed more or less displacement of the S-T segment in several leads during the 50 and 30 mm periods. These electrocardio-

* Supported by a grant from the Commonwealth Fund.

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¹ Wiggers C J, and Werle, J M, *Proc Soc Exp Biol and Med*, 1942, **49**, 604.

² Huizenga, K A, Brofman, B L, and Wiggers Carl J, *J Pharm and Exp Therap*, 1943, **78**, 139.

³ Wiggers Carl J, and Werle J M, *Am J Physiol*, 1942, **136**, 421.

⁴ Wegera, Rene, Guevara Rojas, A, and Wiggers, Carl J, *Am J Physiol*, 1943, **138**, 212.

⁵ Wiggers, C J, *Am J Physiol*, 1945, **144**, 91.

⁶ Green, H D, Dworkin, R M, Antos, R J, and Bergeron, R, *Am J Physiol*, 1944, **142**, 494.

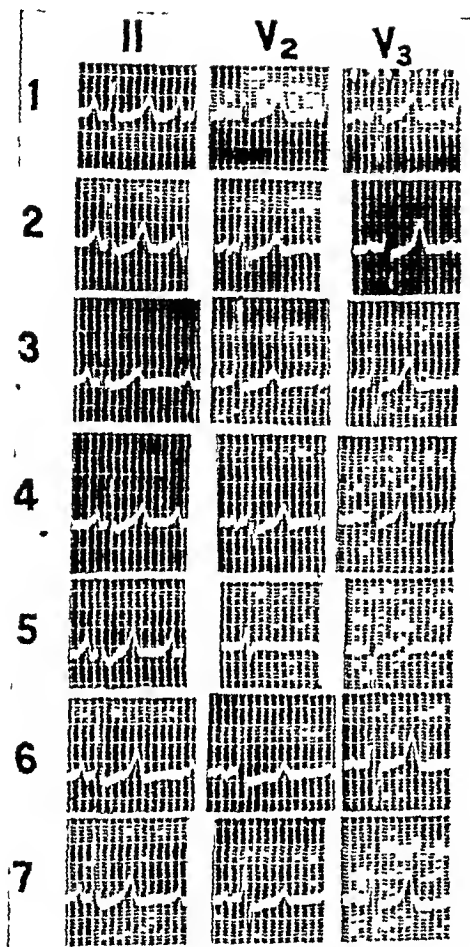


Fig 1

grams generally became normal again immediately after reinfusion, but again developed about the same degree of S-T displacement as arterial pressure began to fall. One dog showed no significant changes at any time. The S-T displacement usually varied from 0.55 to 2.5 mv. The greatest displacement usually occurred in leads V_2 and V_3 , less often in leads II and III (standard), and rarely in lead I. In 5 of the 8 dogs, depression of the S-T segment was much greater during the period of initial hypotension than during circulatory failure following reinfusion and in 2 instances no deviation of the S-T segment occurred during post-infusion circulatory failure.

Sample records of leads II, V_2 and V_3 during the course of one experiment are reproduced in Fig 1. It can be seen at a glance that the character of QRS changes during the course of the experiment. This is not remarkable or significant when we consider that the size and position of the dog's ventricles and their relation to the chest wall change as a result of withdrawal and reinfusion of blood and possible changes in the expiratory position of the thorax.⁷ Whether this also enters into changes in the configuration of T waves in the upper 3 records must remain undecided at the present time. However, it is improbable that displacements of the S-T segment with peaking of the large T waves in records 4, 6 and 7 can be referred to factors other than functional changes in the myocardium. Curves 1 show normal controls, curves 2 and 3 were taken at the beginning and end of the 50 mm period of post-hemorrhagic hypotension. In both series the downward displacement of S-T is significant. Series IV at the end of the 30 mm period still reveals displacement and a peaked T wave, particularly in leads V_2 and V_3 . Series V, taken within 15 minutes after reinfusion, shows no significant changes in QRS. The T waves are small and S-T depression is absent in the chest leads. Series VI was taken about $\frac{1}{2}$ hour after reinfusion, while arterial pressure was still sustained. Marked depression of the S-T segment redeveloped particularly in leads V_2 and V_3 , and T becomes larger and peaked in chest leads. Series VII was taken during the subsequent circulatory failure when arterial pressure had declined to about 60 mm Hg. Persistence of S-T depression and the large peaked T is obvious.

Conclusions Constant displacement of the S-T segment in several leads (one exception) during the periods of severe hypotension indicates that functional myocardial changes occurred as a result of reduced coronary blood flow. The persistence of such changes after reinfusion, or their reappearance *before* arterial pressure has declined a great deal, strongly support the concept that functional

⁷ HARRIS, A. S., *Am J Physiol*, 1945, **143**, 140

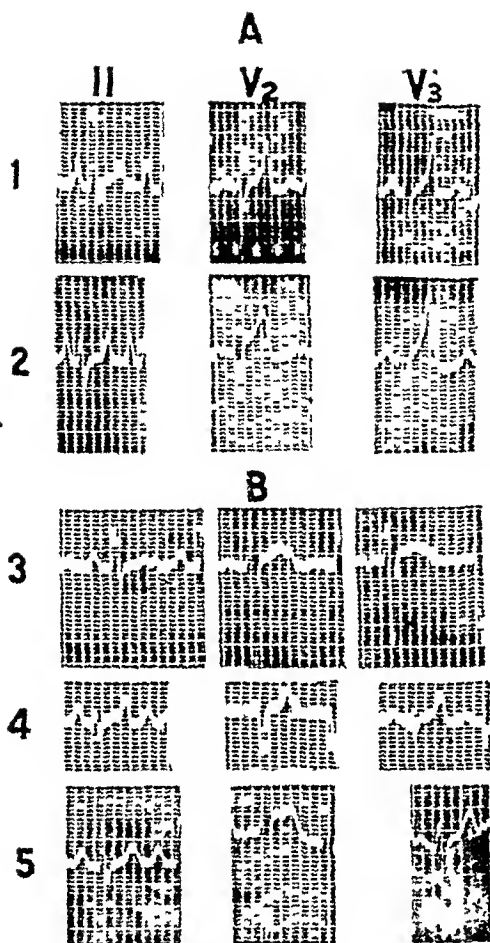


Fig. 2

myocardial changes contribute to the subsequent circulatory failure

Results in Ischemic Compression Shock
In 9 animals, to our surprise, significant S-T deviations generally appeared in several leads during the 6-hour period when compression tubes were kept on and while hypertension (140-210 mm Hg) existed. In 3 dogs the S-T segment was elevated and in 6 depressed (0.5-2 mv) (In one dog the S-T segment reverted to normal and in another it increased progressively). Although dogs were fully anesthetized, painful stimulation was indicated by the augmented breathing and other reactions. It appears that such reflex stimulation in some way affects the myocar-

dium, as indicated by S-T displacement. This is supported by the fact that upon removal of the tubing and release from painful stimulation, the S-T displacement was decreased or abrogated in 7 dogs. However, the displacement of S-T increased in 2 animals. As circulatory failure developed, the displacement increased in one dog only, in the others it remained nearly the same. If these electrographic signs are diagnostic of myocardial impairment it appears that the damage generally occurs during compression rather than after its release. As in hemorrhagic shock, the changes in T waves consisted chiefly of an increase in amplitude of T deflections and sharper peaks; inversion was never observed.

Typical changes in leads II, V₂, and V₃ from 2 animals during compression and decompression are reproduced in Fig. 2. In record 1 taken during compression, slight displacement of S-T is seen in leads II and V₂. Upon decompression, shown in record 2, the displacement becomes very marked in leads V₂ and V₃. Such reactions occurred in 2 dogs only. The typical effects in the other dogs are shown in curves 3 and 4. In curve 3 taken during compression, definite elevation of S-T is shown in leads V₂ and V₃. After decompression (curve 4) this displacement is abrogated.

In this connection the observations of Cicardo⁵ that the plasma potassium of femoral venous blood is definitely increased immediately after removal of a tourniquet (avg 11.2 < 15.2 mg %), require correlation. The fact that characteristic displacements in S-T segments are not generally increased, but more often reduced after removal of the rubber tubes, suggests that the increased quantities of potassium which may enter the circulation after decompression are not always sufficient to be detectable by electrocardiographic leads.

Effects of Massage Since the electrocardiographic changes during the course of circulatory failure did not alter significantly, the procedure was altered in the latter experiments by massaging the swollen limbs

⁵ Cicardo, V. H., *Rev. d. l. Soc. Arg. de Biol.*, 1943, 19, 511.

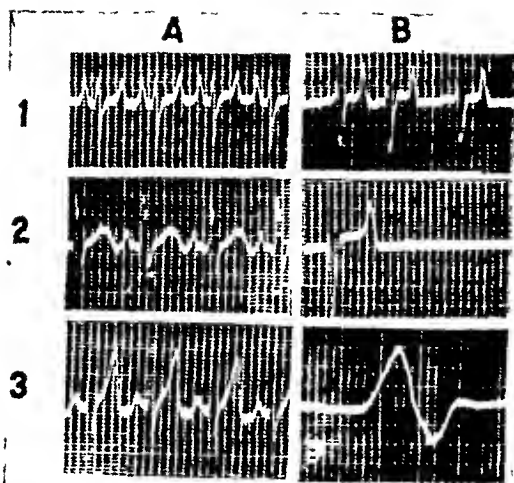


Fig 3

It was obvious from observations of arterial pressures that this hastened the downward course materially. As a rule, each period of massage decreased the heart rate materially. Thus, in several animals the following decelerations were observed $204 > 160$, $136 > 96$, $220 > 180$, $160 > 60$ per minute. A drop of blood pressure attended each reduction in heart rate. Electrocardiograms showed a definite increase in S-T segment displacement as illustrated in curve 5, Fig 2. One dog developed atrial fibrillation 32 minutes after massage, another showed a pronounced elevation of the S-T segment, and a third passed into ventricular fibrillation.

Fig 3 illustrates changes in V_3 leads of 3 different animals. Record 1 shows atrial fibrillation with marked peaking of T waves, record 2 shows bradycardia of a dying heart, and record 3 broad deflection preceding ventricular fibrillation. Attention is called to the fact that ventricular fibrillation does not develop from a series of progressively accelerating beats, as after electrical stimulation or coronary occlusion, but is preceded by broad deflections similar to those reported as preceding fibrillation due to K or digitalis.^{9,10}

⁹ Wiggers, Carl J, *Am J Physiol*, 1930, 93, 197

¹⁰ Wegerin, R, Geyer, J H, and Brown, B S, *J Pharm and Exp Therap*, 1941, 71, 336

We may therefore conclude on the basis of our experiments that while absorption of substances with a potassium-like action is not always detectable by electrographic technics when limbs are kept at rest, such substances are obviously present in tissues submitted to prolonged ischemic compression, and can be pressed into the circulation by massage (and possibly movements) in sufficient amounts to accelerate the production of circulatory failure. Electrographic studies strongly suggest that cardiac damage plays an important role, and indeed may be a cause of sudden death after massage. The importance of complete rest of damaged limbs is reemphasized.

Summary Changes in configuration of records from chest leads paired with a central terminal and of the 3 standard electrocardiographic leads were studied during the course of standardized hemorrhagic shock and ischemic compression shock produced in dogs.

During post-hemorrhagic hypotension (50-30 mm Hg) displacement of the S-T segment in several leads occurred in 8 out of 9 animals. The greatest displacement usually occurred in leads V_2 and V_3 , it occurred less often in leads II and III, and rarely in lead I. The electrocardiograms became normal again after replacement of withdrawn blood, but S-T displacement redeveloped and the T waves became large and sharp before arterial pressure had started to fall. The electrographic changes support the concept that myocardial changes develop in hemorrhagic shock. Similar changes in T wave and S-T segment in several leads—dominantly in V_2 and V_3 —also occurred in ischemic compression shock. The alterations occur soon after limb compression by rubber tubes. A reflexogenic origin is postulated. In 7 out of 9 dogs displacement of the S-T segment decreased materially, or was completely abolished on removal of the compression tubes. The displacement increased in only one animal with development of circulatory failure. However, a prompt, marked increase in the S-T segment occurred when a bruised limb was massaged, and various serious cardiac disturbances (marked slowing, atrial and

ventricular fibrillation) developed after vigorous massage. The results suggest that toxic substances with potassium-like actions are present in damaged tissues, that they do not enter the blood stream in sufficient

amounts to be detectable when limbs are kept at rest, but that they may contribute materially to the circulatory failure and even lead to prompt cardiac death whenever limbs are massaged.

15336

Respiration of Heart Muscle Slices from Rats in the Terminal Stage of Hemorrhagic Shock *

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Although venous return, cardiac output, and blood pressure are diminished in shock, it is usually assumed that the condition of the heart muscle plays no part in the development of severe states of shock. Reflex dilatation of the coronary vessels and the consequent re-routing of larger amounts of blood through the lungs tend to favor the oxygen supply of heart muscle. However, a period of relative anoxia of the cardiac muscle in the early stages of shock after hemorrhage may lead to an impaired ability of the muscle to consume oxygen. A fall in the rate of energy production of the heart muscle might, therefore, play a part in the reduction of cardiac output and of blood pressure seen in shock. The possibility that the rate of respiration of heart tissue, like that of liver tissue,¹ is depressed in hemorrhagic shock in the rat has, therefore, been examined in a short series of experiments.

Methods. Shock was produced in male rats of the Sprague-Dawley strain, weighing between 220 and 250 g, by slow hemorrhage from the tail vein and subsequent replacement of the blood volume according to the method of Sayers, Sayers, and Long.² In this procedure a prolonged state of severe shock is developed, and the animal dies, even

though more than the original volume of blood lost by hemorrhage is replaced. When each animal was in the last stages of shock, several minutes before death was expected (as indicated by the gasping nature of the respiration), the heart was quickly excised, immersed in physiological salt solution until the blood had been pumped from the ventricles, and the great vessels were trimmed away. Thin slices of the heart muscle were prepared and collected in a moist chamber immersed in ice. Samples of the tissue weighing about 85 mg were distributed in Warburg vessels containing 1.5 ml of Krebs' physiological salt solution buffered with phosphate (0.017 M, pH 7.4). Parallel samples in duplicate were weighed into tared vessels and placed in the oven for drying to constant weight at 110°C. The percentage dry weight so obtained was used to calculate the dry weight of the tissue samples used for the measurement of respiration, and the rates of oxygen uptake were thus calculated in terms of the initial dry weight of the tissue. The oxygen uptake was measured in the Warburg apparatus, at 38°C, under an atmosphere of 100% oxygen, using 4 vessels, 2 without and 2 with added substrate. The substrates used were glucose (200 mg %) and sodium pyruvate (0.02 M). Addition of

* The work described in this paper was done under a contract, recommended by the Committee on Medical Research, between the Office of Scientific Research and Development and Yale University.

¹ Russell, J. A., Long, C. N. H., and Wilhelm, A. E., *J. Exp. Med.* 1944, **79**, 23.

² Sayers, G., Sayers, M. A., and Long, C. N. H., unpublished observations.

TABLE I

Rates of Oxygen Uptake of Heart Muscle Slices from Normal Rats and from Rats in the Terminal Stages of Prolonged Severe Hemorrhagic Shock, in the Absence of Substrate and in the Presence of 0.02 M Sodium Pyruvate

Substrate	Group	No of animals	No of determinations		Minutes		
					30	60	90
None	Control	9	18	Mean Q_{O_2} *	$7.86 \pm 34 \dagger$	7.9 ± 3	7.14 ± 29
	Experimental	5	10	P	02	05	01
Sodium Pyruvate (0.02 M)	Control	7	14	Mean Q_{O_2}	5.74 ± 34	6.22 ± 39	4.82 ± 38
				Mean Q_{O_2}	9.13 ± 35	11.3 ± 4	10.59 ± 24
				P	01	01	03
	Lap	5	10	Mean Q_{O_2}	7.61 ± 36	9.7 ± 36	9.85 ± 35

* Cumulative oxygen consumed per mg of initial dry weight per hour

† Standard error of the mean

0.5 ml of substrate solution or of physiological salt solution from the side bulbs of the vessels was made after an equilibration period of 10 minutes. Control observations were made on tissue slices prepared from the hearts of normal rats anesthetized with nembutal (4 mg per 100 g of body weight).

Results and Discussion In 2 preliminary experiments it was found that added glucose depressed the oxygen consumption of normal cardiac muscle slices. There was a uniform elevation of the rate of oxygen uptake when sodium pyruvate was used as a substrate, and the remainder of the experiments were carried out in duplicate with this substrate. It was found that no additional information was obtained when the incubation was prolonged beyond 90 minutes, the decrement in rate of respiration being similar for cardiac muscle slices both with and without substrate. There was no significant difference in the initial dry weights of heart tissue samples from control and experimental animals. The percentage in the former was 22.1 ± 2.2 and in the latter it was 20.9 ± 2.9 .

The results of a series of observations on control animals and on animals in the terminal stages of hemorrhagic shock are summarized in Table I. The rate of oxygen uptake, in the absence of substrate, of heart muscle slices from rats in shock is significantly less than the normal rate. This difference increases with increasing time of incubation, indicating that the respiration is also less well maintained than it is in heart muscle slices from normal rats. In the terminal stages of shock after hemorrhage, the rate

of respiration of the heart muscle is, therefore, moderately depressed. If, however, this effect is to play a part in the development and maintenance of shock, it must occur at a much earlier time. Further experiments are necessary to determine how soon such an effect on cardiac respiration may develop.

The rate of oxygen uptake of heart muscle slices from rats in shock, in the presence of sodium pyruvate, is also less than the normal rate, but the observed differences in rate are not at any time significant. There is therefore, no conclusive evidence of impaired ability of the heart muscle to utilize pyruvate or to maintain respiration at the higher rate attained after the addition of this substrate. It is possible, however, that a real difference in the rate of oxygen uptake of heart muscle from rats in shock and from normal rats might become apparent with a saturating concentration of pyruvate.

These preliminary observations on cardiac tissue metabolism in the terminal stages of hemorrhagic shock indicate that a further study of changes in cardiac metabolism during shock might be profitable. It is desirable to repeat these experiments on animals in earlier stages of shock and to supplement the studies on respiration with determination of rates of glycolysis and of the lactic acid and glycogen content of the heart muscle of animals in various stages of shock.

Summary The rate of oxygen uptake of heart muscle slices from rats in the terminal stages of prolonged severe hemorrhagic shock is significantly less than normal in the absence of substrate. In the presence

of pyruvate (0.02 M) the rate of oxygen uptake is increased, but is still less than the normal rate. There is, therefore, no con-

clusive evidence of impaired ability of the heart muscle to oxidize pyruvate in these circumstances.

15337

Thiamin Deficiency and Susceptibility of Rats and Mice to Infection with *Salmonella typhi murium**

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It has been shown that rats and mice which are deficient in either riboflavin, vitamin A or biotin are markedly susceptible to infection with *Salmonella* (riboflavin Kligler, Guggenheim and Buechler,¹ vitamin A Kligler, Guggenheim and Henig,² biotin Kligler, Guggenheim and Herrnheiser)³ In vitamin A deficiency the resistance falls noticeably in late stages of the avitaminosis and is similar in extent to that of animals given adequate amounts of vitamin A but allowed only as much food as is ingested by animals on vitamin A-free diet. In riboflavin deficiency, on the other hand, a specific effect on the susceptibility of the animals to *Salmonella* is achieved. The susceptibility of riboflavin-deficient mice to *Salmonella* infection exceeds both that of paired isocaloric-fed mice on full vitamin diet and that of normal control mice. The effect of biotin deficiency is an even more specific one. In this case the increased susceptibility precedes the fall in the food intake and cannot, therefore, be due even partially to the latter.

Below we present experiments concerning the effect of thiamin deficiency on the susceptibility of rats and mice to *Salmonella* infection.

* These studies were supported by a grant from the Palestine Endowment Fund, New York.

¹ Kligler, I. J., Guggenheim, K., and Buechler, E., *Proc. Soc. Exp. Biol. and Med.* 1944, **57**, 132.

² Kligler, I. J., Guggenheim, K., and Henig, E., *J. Hyg.* 1945, **44**, 61.

³ Kligler, I. J., Guggenheim, K., and Herrnheiser, H., *J. Inf. Dis.*, in press.

Methods. The rats and mice were taken from our breeding stock after weaning. The experimental diet consisted of 72 parts rice flour, 13 parts casein, 8 parts dried yeast and 4 parts salt mixture. After autoclaving for 6 hours at a pressure of 15 lb. 3 parts olive oil and 2 mg. calcium pantothenate per 100 g. food were added. Each rat was given 100 I.U. vitamin A and 4 I.U. vitamin D twice a week, the mice receiving half this amount. The control and paired-fed rats received in addition daily 20 γ thiamin, the control and paired-fed mice received 10 γ of thiamin daily.

The strain of *Salmonella typhi murium* employed for the production of the experimental infections was the same as that used in earlier experiments.

Results. I. Experimental infections of rats. Forty-eight animals were divided into 3 groups. Group A received food *ad libitum* without supplement of thiamin; group B was paired-fed with group A and received a supplement of thiamin; group C received food *ad libitum* and supplement of thiamin. The ingestion of food in group A was in the first 2 weeks of the experiment normal but from the third week began to fall off gradually, until it rated in the fifth week of the experiment only one-half that of group C. The weights became constant at this stage. As rats of group A reached this stage of avitaminosis, all 3 groups were infected *per os* with 0.1 cc. of a 24-hour broth culture of *Salmonella typhi murium* diluted 1:10 with milk. Three days later the animals were killed, and cultures were made from their

TABLE I
Susceptibility of Rats in Various Stages of Thiamin Deficiency to *Salmonella typhi murum* Infection

Nutritional condition	No of rats	Weeks of experimental diet	Wt in g		No infected	No of rats with infection in			
			At start of exper	At time of infection		Liver	Spleen	Liver and spleen	Mesenteric glands
Deficiency	16	4.5	27	44	15	13	11	11	14
Paired fed	16	"	27	61	15	13	13	13	13
Control	16	"	27	90	9	3	4	2	7
Deficiency	30	2	27	38	3	1	2	0	2
Control	30	"	27	53	3	1	1	1	2

liver, spleen and mesenteric glands. The results are given in Table I. The thiamin-deficient and paired-fed rats proved markedly less resistant to *Salmonella* infection than the controls. Statistically this diminished resistance was found to be highly significant. The decrease of resistance was reflected both in the incidence and in the manner of distribution of the infection.

The similar response of the paired-fed and of the avitaminotic group shows the non-specific nature of the effect of thiamin deficiency. This is confirmed by the experiments in which rats in an early stage of avitaminosis (after 2 weeks of the deficiency diet) were infected, *ie*, before any decrease in food intake had occurred. As may be seen from Table I, hypovitaminotic rats are not more susceptible to infection than the controls.

II Observations on experimental epidemics in mice. Four-week-old litter mates were taken directly from the mothers and divided into 3 groups, each group comprising 60 mice. Group A was kept on our thiamin-deficient diet, which was given *ad libitum*, group B was isocalorically paired fed to A with thiamin supplement, whereas group C received food *ad libitum* with thiamin supplement. Each group was divided into 4 cages, 15 mice per cage. At the start of the experiment one mouse in each cage was infected with 0.05 cc of a 24-hour broth culture of *Salmonella typhi murum*. Cultures were made from the liver, spleen and mesenteric glands of mice which died during the following fortnight. At the termination of this observation period the survivors were killed and examined similarly. The results are summarized in Table II. The table shows that the gain of weight of mice kept on a thiamin-deficient diet is significantly below that of the 2 other groups, although the food intake of the paired-fed group and of the control mice was practically the same. It is also evident that the thiamin-deficient mice showed a markedly higher rate of infection than the paired-fed and control mice. This enhanced susceptibility to infection on the part of the thiamin-deficient mice can only be attributed, therefore, to their thiamin

TABLE II
Susceptibility of Mice in Various Stages of Thiamin Deficiency to *Salmonella typhi murum* Infection

Nutritional condition	No. of mice*	Period of observation, days	Wt in g		No. alive and cultured	Total infected†	No. of mice with infection in			
			Start of experi	At time of cul- turing			Liver	Spleen	Liver and spleen	Mesenteric glands
Deficiency	56	15	11.7	12.5	36	20 (100)	16 (80)	16 (80)	17 (75)	20 (100)
Partial fed	56	15	11.7	14.0	9	11	14	15	9	15
Control	56	15	11.7	14.1	8	17 (66)	14 (30)	17 (32)	10 (17)	32 (51)
Deficiency	80	8	11.2	11.6	1	59 (77)	6 (24)	8 (36)	6 (21)	56 (67)
Control	80	8	11.2	12.4	0	10 (75)	10 (8)	9 (10)	6 (8)	17 (71)
						50 (50)	12 (12)	11 (11)	8 (8)	46 (46)

* The one initially infected mouse in each cage is not included in the number given.

† Out of a total of 54 mice which died the organs of 42 mice were cultured. In every case general infection with *Salmonella* was found.

‡ The numbers in parentheses show the percentage.

deficiency

A feeble effect of thiamin deficiency is already demonstrable, as the following experiment shows, on the eighth day of the observation period 84 litter mates were divided into 2 groups. The animals were kept in cages which contained 21 mice each. One mouse in each cage was infected, as described above and the entire mouse population was then treated in the manner of the former experiment, with the exception that the survivors were killed after 8 days. As may be seen from Table II, a difference in the incidence of infection in the thiamin-deficient group is already demonstrable at this early date. It is evident, however that the mouse organism is still able at this stage to combat the invasion of bacteria from the mesenteric glands.

Discussion The experiments and observations reported above provide an unequivocal answer to the question, whether thiamin deficiency affects resistance to *Salmonella* infection. When rats and mice are placed on a thiamin-deficient diet, they evince markedly increased susceptibility to enteric infection already within a few weeks.

There is a significant difference between the behaviour of the rat and of the mouse. Whereas in the case of the mouse the diminished resistance is exclusively attributable to the thiamin deficiency, the higher susceptibility of rats is due to the inanition which accompanies avitaminosis. The mice behave, in other words, as in the case of biotin deficiency,³ whereas the rats behave as in the case of vitamin A deficiency.²

The effect of thiamin deficiency on resistance to infection has been studied by several authors. Badger, Masunaga and Wolf⁴ observed a diminished resistance to rat leprosy in thiamin deficiency. A high susceptibility to experimental pneumococcal infection could be shown by Wooley and Sebrell⁵ in mice and by Robinson and Siegel⁶

⁴ Badger, L. F., Masunaga, E., and Wolf, D., *Pub. Health Rep.*, 1940, **55**, 1027.

⁵ Wooley, J. G. and Sebrell, W. H., *Pub. Health Rep.* 1942, **57**, 149.

⁶ Robinson, H. J. and Siegel, H., *J. Inf. Dis.*, 1944, **75**, 127.

in rats. The diminished resistance was found to be due to the avitaminosis *per se*, the concomitant starvation having no apparent influence. Thiamin-deficient rats show also a lowered resistance to infection by *Nippostrongylus muris*⁷. On the other hand, thiamin-deficient mice exhibit an increased resistance to infection with Theiler's virus and poliomyelitis⁸⁻¹¹. Clark, Waisman, Lichstein and Jones¹² showed, however, in

studies with *Macaca mulatta*, that this species, when deficient in thiamin, does not exhibit increased resistance to poliomyelitis virus. Further study of the effect of thiamin deficiency on the resistance of different species of animals to bacterial and viral infection seems, therefore, to be necessary.

Summary (1) Rats and mice kept for a few weeks on a thiamin-deficient diet show diminished resistance to oral *Salmonella typhimurium* infection.

(2) In rats the lowered susceptibility is secondary to inanition. On the other hand, in mice the diminished resistance to *Salmonella* infection is a primary result of the thiamin deficiency.

¹² Clark, P. F., Waisman, H. A., Lichstein, H. C. and Jones, E. S., *Proc Soc Exp Biol and Med*, 1945, **58**, 42.

⁷ Witt, J. Y. C. *Am J Hyg*, 1944, **39**, 145.

⁸ Foster, C., Jones, J. H., Henle, W., and Dorfman, F., *Am J Med Sc*, 1943, **205**, 465.

⁹ Foster, C., Jones, J. H., Henle, W. and Dorfman, F., *Science*, 1943, **97**, 207.

¹⁰ Foster, C., Jones, J. H., Henle, W., and Dorfman, F., *J Exp Med*, 1944, **80**, 257.

¹¹ Rasmussen, A. F., Waisman, H. A., Elvehjem, C. A. and Clark, P. F., *J Inf Dis*, 1944, **74**, 41.

15338

Rh Antigen and Hapten. Nature of Antigen and Its Isolation from Erythrocyte Stroma

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Introduction The demonstration of the role played by the Rh factor of human erythrocytes in isommunization^{1,2} leads to speculation concerning the usefulness of the isolated antigen or hapten in modification of the sensitized state. This would be particularly important in hemolytic disease of the newborn, in which maternal anti-Rh antibodies enter the foetal circulation and produce accelerated hemolysis of the infant's erythrocytes. It seems possible that a preparation of Rh antigen could be used to neutralize the anti-Rh antibodies in the maternal circulation and tissue. If sufficient

antigen could be injected at regular intervals antibody formation, which apparently has some natural limitation, could be overwhelmed and the subject kept in a state of desensitization until pregnancy is terminated. If the isolation and purification of the Rh antigen can be carried to the point of separation of the Rh hapten from the associated protein it would presumably no longer possess antigenic qualities but would still be capable of neutralizing the antibodies.

Belkin and Wiener³ have shown by serum inhibition experiments that the Rh factor as well as the A and B substances can be demonstrated in roughly quantitative amounts in the stroma of human erythrocytes. We

¹ Wiener, A. S., and Peters, H. R. *Ann Int Med*, 1940, **13**, 2306.

² Levine, P., Katzin, E. M., and Bunkam, L. J. *J M A*, 1941, **116**, 825.

³ Belkin, R., and Wiener, A. S., *Proc Soc Exp Biol and Med*, 1944, **50**, 214.

TABLE I
Selective Inhibition of Anti Rh₀ Serum by Stroma from Rh Positive Cells

Dilutions of 10% stroma suspension	11	12	14	18	116	132
Stroma from Rh+ cells	0	0	0	0	+	+
Stroma from Rh- cells	+	+	+	+	+	+

have undertaken to find means of separating the Rh antigen from other components of erythrocyte stroma by chemical and physical methods and to obtain information as to the nature and properties of the antigen

Material and Methods 1 *General methods for production of post hemolytic residues or stroma* The erythrocytes used were obtained from citrated blood after centrifugation and removal of the plasma*. Usually only group O cells were used, but on some occasions cells from 50 to 100 untyped bleedings were pooled without regard to blood group. On some occasions stroma was prepared from Rh positive and from Rh negative cells, but in general large lots of cells were used without regard to Rh typing. Most of the leucocyte and platelet layer was removed from the centrifuged blood before the cells were hemolyzed.

Hemolysis was accomplished by adding 8 to 10 volumes of distilled water which had been chilled to a temperature of 5°C. In the initial preparations the solution was then fed through a Sharples supercentrifuge. The rate of flow depended on the model and speed of the centrifuge employed. The red jelly-like material collected by centrifugation was sometimes resuspended and sedimented again in order to remove more of the remaining hemoglobin.

The material was then kept in solution at a pH between 7 and 8 or dried by lyophilization. In either state it was found to retain most of its potency for weeks, when stored at 2°C.

Suspensions of the dried material, or fractions thereof, were made in 10% concentration in isotonic saline, or in phosphate buffer pH 7.7 ionic strength 2. An even suspension of fine particles was obtained by

grinding the suspension in a mortar. In later purified preparations the material dispersed quite readily upon mixing and standing a few hours.

2 *Methods used in detecting the presence and concentration of the Rh antigen in erythrocyte stroma and fractions thereof* The anti-Rh serum used in the inhibition experiments agglutinates approximately 85% of human Rh-positive bloods and has a titer of 400. It was used in dilution of 1-20 with normal saline.

Serial dilutions were made of the stroma suspensions, which were then mixed with equal volumes of the constant dilution of the anti-Rh serum. The stroma serum mixtures were incubated for one hour at 37°C. Following incubation the tubes were centrifuged to obtain a relatively clear supernatant fluid. One drop from each tube was incubated for 1/2 hour with one drop of a 2% suspension of Rh positive cells which had been shown to give a strong reaction with the anti-serum. Final observation for the presence or absence of agglutination was made after centrifugation and inspection of the erythrocytes under low power magnification. The inhibition titer of the stroma preparation was read as the highest dilution of a 10% suspension which inhibited agglutination by the constant strength of the anti-Rh serum.

The same technic was used in quantitating the A and B substances in stroma preparations made from pooled untyped cells, except that a constant strength of groups B and A sera was used. The sera had titers of 3000 and were used in dilutions of 1-20.

Inhibition of anti-Rh serum with stroma from Rh-positive and Rh-negative erythrocytes Our first experiments were designed to demonstrate that the inhibition of anti-Rh serum by stroma from Rh-positive cells was not due to a nonspecific effect. Typical results of such observations are recorded in Table I.

* We are grateful to the Cutter Laboratories of Berkeley, California, and especially to Mr. Virgil Herring, for the red cells and for the first stages in their processing.

TABLE II
Sensitivity to Destruction of Rh Factor in Stroma by Heat

Dilutions of 10% stroma	1 1	1 2	1 4	1 8	1 16	1 32	1 64
Control (unheated)	0	0	0	0	0	+	+
56°C 15 min	+	+	+	+	+	+	+
100 15 "	+	+	+	+	+	+	+

TABLE III
Heat Destruction of Rh Factor with Moisture in Absence of Oxygen

Dilutions of stroma (10%)		1 1	1 2	1 4	1 8	1 16
Control	no heat	0	0	0	+	+
Tube No 1, vacuum	56°C 5 min	0	0	0	+	+
" No 2, air	" 5 "	0	0	0	+	+
" No 3, moisture	" 5 "	+	+	+	+	+

Observations on the stability of the Rh factor in Erythrocyte Stroma 1 Heat stability of the Rh factor in stroma One of the characteristics of an antigen is its thermal stability (or instability). The results of such tests on the Rh antigen are given in Table II.

Further observations have indicated that the antigen is completely inactivated according to our methods of testing by exposure to 56°C for 5 minutes.

Stroma preparations containing in addition to Rh also the factors inhibiting group A and B sera showed no loss of these properties upon heating when the Rh antigen had been completely inactivated.

2 Effect of the presence of an and moisture on the heat stability of the Rh antigen Aliquot portions of active stroma were lyophilized and sealed in 3 small evacuated tubes. The first tube was kept evacuated, while the contents of the second were exposed to air. To the third tube, the requisite amount of saline was added without breaking the vacuum. The saline had been previously boiled to remove dissolved oxygen, and then cooled.

All 3 tubes were then exposed to 56°C for 5 minutes. The results of serum inhibition experiments with samples from these tubes are shown in Table III.

The Fractionation of the Stroma Red cell stroma suspensions have been prepared in a variety of ways with a corresponding variety of results.^{4 5 6} It was soon apparent

to us that because of the extreme sensitivity of the Rh antigen, chemical methods of fractionation would not be as satisfactory as physical methods, at least in the early stages of the separation.

Following the method of Jorpes,⁶ we prepared the stroma suspensions by adjusting the pH of the original solution of hemolyzed red cells to 5.5-6.0, at which a heavy, easily separated precipitate was formed. This was washed several times at pH 5.5-6.0 to remove most of the adsorbed hemoglobin. When it was redissolved at pH 7-8 we found that a cloudy solution was produced from which no sediment would settle upon standing. However, when this solution was centrifuged at 50,000 G a material (elmin) was sedimented, leaving a clear supernatant from which a heavy precipitate could again be thrown out by readjustment of the pH to 5.5. It corresponds to the protein which Jorpes has called stromatin, which name we retain. Stromatin so prepared exhibits no inhibition of the anti-Rh serum.

The material (elmin) removed from the above mentioned cloudy solution by centrifugation at 50,000 G dissolves easily at pH 7.5-8.0, producing a milky solution which shows very marked streaming double refraction. Since this material is very different from stromatin and can be characterized by its physical, chemical and biological properties we propose to call it elmin.

The approximate yield of stroma was about 1-2 g per 250 cc of red cells, of which 35 to

⁴ Ponder, E., *The Mammalian Red Cell and the Properties of Hemolytic Systems*. Berlin: Borntraeger, 1934.

⁵ Segundsson, B., *J. Exp. Med.*, 1943, **77**, 315.

⁶ Jorpes, E., *Biochem. J.*, 1932, **26**, 1488.

40% is elinin and the rest stromatin

The ability to inhibit the anti-Rh serum was found only in the elinin fraction which also possesses a concentration of the A and B substance 4 to 5 times greater than does stromatin

The crude stroma suspension contains about 20% lipids extractable by 3:1 alcohol ether. The elinin contains 40-50% lipids extractable by the same solvent. The nitrogen and phosphorous analyses are as follows

	Prep I		Prep II	
	N %	P %	N %	P %
Stromatin	12.3	0.42	12.1	0.25
Elinin	9.1	1.0	8.1	1.0

Neither of these preparations can yet be said to be pure

Electrophoresis[†] of a 1% solution of elinin at 2°C in phosphate buffer pH 7.8 ionic strength 2, showed a main component (about 80% of the total) having an average mobility of 6×10^{-5} cm² volt⁻¹ sec⁻¹ (negatively charged), and a faster component (about 15% of the total) having a mobility of about 8×10^{-5} . The partial specific values of elinin at 20° is about 0.83

The samples of elinin so far prepared have exhibited inhibition titers for the anti-Rh serum as high as 1:32. On a number of occasions we have succeeded in separating from the elinin an ether-soluble material which exhibited inhibition titers for the anti-Rh serum as high as 1:128. But heretofore we have not been able to reproduce this extraction consistently. The ether-soluble

factor appears to be relatively thermostable, i.e., it is not inactivated by 60 minutes heating at 56°C. Part of the original thermostability of elinin is returned upon recombining the protein from which the ether-soluble material had been removed, with the ether-soluble fraction.

When elinin is prepared from pooled untyped blood it exhibits relatively high inhibition titers (1:512) for the standard anti-A sera and a correspondingly lower inhibition titer (1:32) for the standard anti-B sera. In the ether-soluble fraction from elinin the inhibition of anti-A serum was reduced (1:16) while the inhibition of anti-Rh serum was increased.

Summary 1 It has been demonstrated that the Rh antigen is quite different in character from the A and B substances as found in stroma from human erythrocytes. The Rh factor is particularly sensitive to thermal inactivation and is destroyed by a few minutes exposure to 56°C while the A and B substances remain intact. The inactivation by heat in the presence of moisture and the absence of oxygen suggests protein denaturation as the basis for its destruction.

2 Erythrocyte stroma has been fractionated into 2 parts, (1) a protein previously described and called stromatin, and (2) a new lipoprotein which we call elinin. The Rh specificity is associated with the elinin.

3 An ether-soluble fraction has been separated from elinin which is characterized by a higher content of the Rh factor and a lower content of A and B substances. The Rh substance present in the ether solution which inhibits the anti-Rh serum showed an increased thermostability.

[†] The electrophoresis was done by Dr. C. H. Li, of the Institute for Experimental Biology of the University of California, Berkeley, California, to whom we express our thanks.

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⁴ Ponder, E. *The Mammalian Red Cell and the Properties of Hemolytic Systems*. Berlin: Borntraeger, 1934.

⁵ Segundsson, B. *J. Exp. Med.*, 1943, **77**, 315.

⁶ Jorpes, E. *Biochem. J.*, 1932, **26**, 1488.

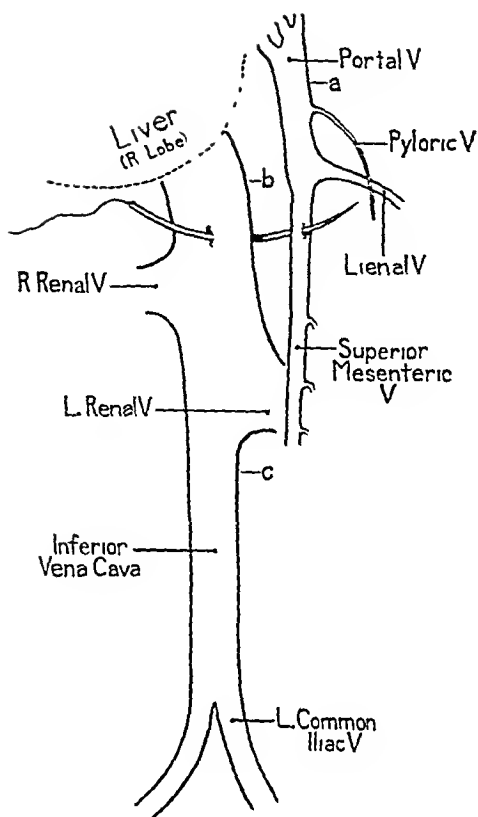


Fig 1

Portal vein ligation and Eck fistula in the rat

solution of cellulose acetate. The thread and hair are well matted together. Heavy adhesive tape is passed around the body covering these areas to prevent the rat from pulling the threads prematurely. A strip of screen wire between layers of tape prevents the removal of the bandage by the animal. Four or 5 days later the tape is removed, the ligature ends exposed and pulled in such a manner that the "surgeon's" knot is tightly closed around the portal vein. This maneuver constitutes the second stage of portal ligation. Should the animal die within a few hours, it is highly probable that the first ligature had been too loose to stimulate development of collateral circulation. The ligature ends may be cut off or by protecting them for 4 more days the thread may be pulled completely from the body after some gentle manipulation. This last procedure was

suggested by the work of Brunschwig.³ This entire operation is practically bloodless. The rat is subjected to little more trauma than that of a laparotomy.

Porto-Caval Anastomosis (Eck fistula)
Well deserved credit is given Nikolai V Eck for developing the first surgical anastomosis between the portal venous system and the inferior vena cava. He, impelled by a desire to study liver function, developed a technic which he applied to 8 dogs. Of the 8, one did not survive 24 hours, 6 lived 2 to 7 days and one recovered completely and lived in the laboratory for 2½ months when, by some negligence it was allowed to escape. Dr Eck's published report gives no indication that he used his dogs in any experimental study nor that he proved the efficacy of the operation but he implies that autopsies of those that died showed patent fistulae in most cases. Apparently military service interrupted his work at that point. At any rate, he believed that he had demonstrated that the operation was feasible, and that life is possible in an animal in which the liver is not the first recipient of the blood from the gastro-intestinal tract. The method was essentially that of securing the 2 veins to each other with 2 longitudinal rows of sutures and then making an incision between them.

Fishback⁴ reviews the literature pertaining to the methods and modifications of the Eck fistula in his report describing the use of a cutting suture in making the anastomosis. Angle⁵ described a special instrument suitable for the dog which clamped the 2 veins without occluding either in such a manner that the anastomosis could be made with a minimum loss of blood. Klages⁶ made the Eck fistula applicable to the rabbit, by causing adhesions to develop between the 2 veins by a first operation and later, by forming a window between them through the adherent

³ Brunschwig, Alexander. *Surgery* 1945 17 781

⁴ Fishback, F. C., *Ann Surg* 1927 86, 436

⁵ Angle, Lewis W. *Am J Surg* n s 1923 19, 347

⁶ Klages, F. *Arch f Exp Path u Pharm* 1928, 188, 489

Portal Vein Ligation and the Eck Fistula in the Rat

WAYNE L. WHITAKER (Introduced by Howard B. Lewis)

From the Department of Anatomy, University of Michigan, Ann Arbor, Mich

Limited though it is, the Eck fistula has been of real value in the study of liver function since its development by N. V. Eck.¹ Many problems of nutrition, detoxication and hepatic inactivation may well be studied by the judicious use of animals in which the blood from the gastro-intestinal tract is diverted from its normal pathway through the liver. Unfortunately the Eck method of producing a porto-caval anastomosis has so far been applicable to only the larger laboratory animals in which even then the surgery has not been easy, and mortality is often high. Furthermore, the inherently proper animal for many problems could not be prepared.

In the search for a method for studying the hepatic inactivation of steroid hormones 2 procedures have been adapted or devised by either of which the widely used laboratory rat may be prepared to function in a manner similar to the Eck fistula dog. These operations require few special instruments and may be done with speed and moderate mortality. The first is a 2-stage ligation of the portal vein. The second is a porto-caval anastomosis comparable to that done on the dog. The 2 methods as well as the "reverse" Eck fistula are described separately.

Portal Vein Ligation If the portal vein is completely ligated at one operation the rat dies of circulatory failure within one or 2 hours, apparently due to the reduced effective blood volume incident to the accumulation of blood in the abdominal viscera. If the vein is only partially ligated, however, at a first operation the increased pressure in the portal system stimulates the development of collateral pathways so that a subsequent complete ligation of the vessel is tolerated. The second similar operation is hazardous because of the severe hemorrhage

resulting from the difficult process of dissecting away adhesions involving intestines and liver. Dragstedt² solved this problem by placing a second ligature at the time of the first operation and securing the ends of the thread on the inner surface of the body wall. His second operation was done easily by opening the abdomen, retrieving the anchored ends of the ligature and pulling the knot down on the vein.

The method here described may be best understood by reference to Fig. 1. An extensive mid-line incision of the abdominal wall is carried to or above the tip of the xiphoid process. With the viscera retracted to the left, the vessels involved are made visible, as illustrated. A thread is passed by curved forceps or the blunt end of a curved needle around the portal vein, at *a* in the figure. Only moderate care is needed to exclude the hepatic artery and common bile duct. A wire about 1 mm in diameter, gauge 16 or 18, is laid along the vein and the ligature formed by the thread which encloses both the vein and the wire is closed firmly. The wire is then withdrawn. The size of the effective lumen of the vein is thus approximately that of the wire employed. This partial ligation is apparently sufficient to stimulate the development of collateral circulation. A second ligature is placed around the vein at the same level and at the same operation. The knot used is the "surgeon's" or friction knot. It is left so loose that it offers no restriction to blood flow. The ends of the thread are carried through the lateral body walls in approximately the same transverse plane as the portal vein. The abdominal incision is closed by a single series of interrupted sutures. The extruding ends of the ligature are secured to the skin surface by a liberal application of an acetone

¹ Eck, Nikolai V., *Travertine meditsinskii Zhurnal*, 1877, 130

² Dragstedt, Lester R., *Science*, 1931, 73, 315

3 or 4 per hour. Efficient assistance can reduce this time considerably. Although surgery is easier on large adult rats, the Eck fistula has been produced on some rats weighing 80 g.

Summary Methods have been described for producing in the rat (a) a simple ligation

of the portal vein and (b) an anastomosis between the inferior vena cava and the portal venous system. By the use of the latter procedure, either a "direct" or an "indirect" Eck fistula may be produced. These techniques should enhance the value of the laboratory rat in liver function studies.

15340 P

Number of Peyer's Patches in Mice Belonging to High and Low Mammary Tumor Strains

MARGARET A. KELSALL* (Introduced by C. C. Little)

From the Roscoe B. Jackson Memorial Laboratory, Bar Harbor, Maine

Peyer's patches are aggregates of lymphoid follicles in the mucosa of the small intestine. Their distribution and structure in the mouse are essentially similar to descriptions given for man with the exception that 1 to 3 patches are usually present in the duodenum of the mouse whereas in man they are found only in the lower part of the small intestine.

A marked difference in the number of Peyer's patches in 2 strains of mice suggested a study to determine whether there was any significant difference in the amount of lymphoid tissue in the small intestine of mice belonging to high and low mammary tumor strains. One of the many functions assigned to the lymphocyte is that it has a part in tumor growth, the explanation of this function ranging from the conclusion that lymphoid tissue is a factor in immunity¹ to the contradiction that it has no part in tumor growth.²

Mice used were of both sexes and varied in age from 4 weeks to 15 years. The animals were not fed for 2 days prior to killing in order that the small intestine might be contracted, thereby making the Peyer's patches more conspicuous. The mice were

killed by etherization, the small intestine and cecum were removed and fixed in a 10% formol solution, for 2 days, after which the intestines were washed thoroughly. The lymphoid nodules comprising the patches make the wall of the intestine considerably thicker, and as a result these areas protrude and are easily visible macroscopically.

The 5 strains may be divided into a low group, having an average of approximately 6.3 Peyer's patches, and including only strain C57 black, an intermediate group, having 8 to 9 patches and including strains C, Swiss, and dba, and a high group including C3H mice, which have a mean of 10.7 Peyer's patches (Table I). The differences between strain C57 black and the other strains were significant according to the χ^2 test for goodness of fit. When data from C57 black mice were used for the theoretical distribution, strains C3H, dba, and Swiss had an χ^2 above 70, the χ^2 for C57 black and C was above 38, which gives a probability of approximately one in a million and indicates that this difference is also significant.

The difference in frequency distribution between strain C3H and the intermediate group is also significant, the χ^2 for differences between C3H and dba being over 70. Additional data are necessary to establish significant differences between dba, Swiss, and C strains. The probability of a difference between dba and Swiss is only one in

* The author is a recipient of a Finney Howell Research Fellowship.

¹ Murphy, I. B., and Morton, J. J., *J. Exp. Med.*, 1915, **22**, 204.

² Sittenfeld, M. J., *J. Cancer Res.*, 1920, **105**, 117.

area Blakemore and Lord⁷ have recently reported the highly successful application of the vitallium tube non-suture technic to the production of a porto-caval anastomosis in a number of patients with severe ascites due to portal bed block.

The anastomosis between the portal venous system and the inferior vena cava here described is done in 2 stages. For the first operation, an extensive mid-abdominal incision is made and the viscera are retracted to the left, exposing the venous systems concerned, as shown in Fig 1. A ligature is placed about the portal vein, at *a* in the illustration, formed with a "surgeon's" knot, but not closed. The ends of the thread are carried through the lateral body walls in preparation for tightening a few days later. In preparation for joining the veins the peritoneum is stripped from the vena cava in the neighborhood of the right renal vein where the vena cava and the superior mesenteric veins may be easily approximated. One of the smallest curved needles available is used to make the anastomosis. An artery suture with attached taper point needle, size 6-0 has been satisfactory, though the small cutting needle commonly found in eye sutures of comparable size offers one advantage. It makes larger punctures in the veins and thus offers more assurance of a successful anastomosis. The increased hemorrhage can be controlled. The needle is passed through the inferior vena cava, as shown in the illustration, and then through the adjacent superior mesenteric vein, piercing the lumina of both, and the suture is tied firmly. The suture includes a substantial part of the walls of the 2 veins. The fistula between the 2 veins should develop here to a functional size within a few days. There is very slight loss of blood from the punctured vena cava but precautions may well be taken to control loss from the mesenteric vein. The abdominal incision is closed and the ends of the delayed portal ligature fixed to the skin and protected as described for the portal ligation operation. Four or 5 days later this previously prepared

ligature around the portal is tightened. If an anastomosis was not produced the animal will die within a few hours.

The "Reverse" Eck Fistula Only a few modifications of the above procedures are necessary to produce the so-called "reverse" Eck fistula by which blood from the inferior vena cava is carried through the liver by way of the portal system. The anastomosis is made in the same manner as above. The delayed ligature is, however, placed on the vena cava above the renal veins (*b* in Fig 1). If it is desired to study the effect of the liver on blood-carried products of the kidneys, adrenals, or gonads more specifically a ligature may be placed, at the time of the first operation, on the inferior vena cava below the renal veins (*c* in Fig 1). This ligature is well tolerated and minimizes the possibility of the return of blood from the above mentioned organs to the heart by general collateral circulation.

The possibility exists that the effectiveness of any of the 3 operations described may be reduced by the post-operative development of venous channels around the ligatures. For example, an adhesion between the intestine and the liver may act as a bridge to carry venous blood directly to the liver. That this is not likely to be a serious hazard is indicated by the striking and generally consistent results already attained in studies of 2 different liver functions by the method. In the "reverse" Eck fistula enlarged collateral pathways around the ligature of the vena cava have been found. How serious this factor may be is not yet apparent. Obviously, a complete evaluation of the procedures described here must await their extensive use in experimental studies.

This single suture method of making a venous anastomosis was first suggested by Dr John Lawther. In fact, he made the first attempt to carry it out.

All manipulations of the animal involved in the above operative procedures have been carried out under ether anesthesia. Non-sterile operative technic is apparently satisfactory for the healthy rat. The time required to do these operations is not excessive, for one operator working alone can perform

⁷ Blakemore, A. H., and Lord, J. W., *Ann Surg*, 1945, 122, 476.

A Quantitative Method for the Study of Chromatolysis *

BERRY CAMPBELL AND ROSALIND NOVICK

From the Department of Anatomy, University of Minnesota

The chromatolytic changes which follow section of the axon and certain other procedures consist in a continuous spectrum of cell alteration from the normal cell to the heavily chromatolyzed and even the ghost cell. To compare the degree of chromatolysis in 2 nuclei or to relate the chromatolytic changes with time, it is imperative that the spectrum of change be broken up into arbitrary and well-defined stages of a convenient number in order that standard quantitative procedures may be applied. Cognizance of this need led Dolley¹ to erect a series of 13 categories into which cells affected by shock and by exercise may be grouped. His method is not suitable for our purpose, however, for it is concerned with nuclear changes as much as with the changes of the Nissl bodies and was designed for rather a different purpose. The following method has been used in this laboratory with success and is recommended as being both simple and adequate.

The sequence of cytoplasmic changes

which constitute the axon reaction is as follows. The Nissl bodies, normally abundant and square-cornered, become rounded, more lightly staining and begin to break down to a fine powder in the central areas of the cell. This fractionation then extends out to the periphery leaving the cytoplasm devoid of any blue-staining substance other than the finely-powdered material. Concomitant with the later stages is a shifting of the nucleus from its normal central position to the periphery of the cell. In addition to these changes, there are frequently present in cell groups in which chromatolysis has been induced, cells which have acquired more heavily-staining properties than the normal cell. These hyperchromic cells which have been noticed by many authors, range from a stage which shows the normal cell structure with the Nissl bodies more heavily stained to pyknotic heavily stained cells in which the cell contents are indistinguishable because of the depth of the stain. The physiological significance of this change is still a matter

TABLE I
Criteria Defining Cell Types

B Pyknosis	A Hyperchromatism	N Normal	1 Mild chromatolysis
Shrunken, intense staining Nucleus and cytoplasm obscured Fig 1,a	Nissl particles larger more heavily staining than normal Fig 1,b	Nissl particles discrete, square cornered Moderately intense stain Fig 1,c	Nissl particles breaking up in center of cell Normal Nissl pattern in periphery Fig 1,d

TABLE I (Continued)

2 Medium chromatolysis	3 Severe chromatolysis
Nissl particles uniformly powdery throughout cell Nucleus central Fig 1,e	Nissl particles uniformly powdery Nucleus peripheral Fig 1,f

* Aided by a grant from the National Foundation for Infantile Paralysis, Inc.

¹ Dolley D. D., *J Med Res*, 1911, 20, 285

of conjecture. From our experience we are led to view such hyperchromic cells as bearing an opposite relation to the normal cell as does the chromatolyzed, and have so dealt with it in the categorization to be described below. It should be noted that Dolley (loc cit) follows the view, that the hyperchromic cell is undergoing changes preliminary to chromatolysis. For this possibility we can find no evidence in our ob-

TABLE I
Range and Frequency Distribution in the Number of Peyer's Patches in the Small Intestine
of 5 Inbred Strains of Mice

Strain	No of Peyer's patches										Total	Mean	St dev	St error
	4	5	6	7	8	9	10	11	12	13				
C57Blk	28	52	68	74	25	18	3	0	1	0	249	6.3	1.5	.09
C	1	2	15	24	24	21	14	8	2	1	112	8.2	1.7	.16
Swiss	2	6	8	34	36	20	21	11	3	0	138	8.3	1.7	.15
dba	0	2	9	22	55	28	20	13	4	3	157	8.6	1.6	.13
C3H	0	0	1	1	6	20	25	26	18	13	124	10.7	1.5	.14

a 100. The means for strains Swiss and C were 8.3 and 8.2 respectively, a difference that is insignificant, with a probability of only one in 10.

No differences were found between sexes within a strain, and the only difference in size observed was that most patches in the C3H mice were larger.

A direct correlation exists between the amount of intestinal lymphoid tissue, as measured by the number of Peyer's patches, and the incidence of spontaneous mammary tumors. Strain C3H has an incidence of mammary tumors in approximately 95% of the breeding females. Strain dba, which follows C3H in the number of Peyer's patches with a mean of 8.0, has a tumor incidence of approximately 51% in virgin females and 85% in breeding females.³ Strains C and Swiss have fewer Peyer's patches and a lower incidence of mammary tumors. In strain C57 black, mammary tumors are extremely rare.⁴

There may be no functional relationship between the Peyer's patches and the mam-

mary gland, however, several facts have been established that prove there is a functional relationship between the lymphoid tissue of the body as a whole and lactation. Emmel, Weatherford, and Streicher⁵ have shown that lymphocytes pass in great numbers from the blood into the ducts of the mammary gland during lactation in the rat. In addition, the reticulo-endothelium of the lymphoid organs, including that in the Peyer's patches, is a source of monocytes, cells considered to be the colostrum corpuscles when occurring in the milk and containing fat globules. Quantitative differences in lymphocytes and monocytes may contribute to tumor growth, but functional differences in the lymphoid systems as a whole need to be established before any conclusions are warranted.

Summary. Line C57 black mice has a mean of 6.3 Peyer's patches, strains C, Swiss, and dba, means from 8 to 9, and strain C3H, a mean of 10.7. Differences between these groups are significant, according to the X^2 test. There is a direct correlation between the number of Peyer's patches and the incidence of spontaneous mammary tumors in these 5 pure lines of mice.

³ Little, C. C., *Biol. Lab. Mouse*, 1941, 248, Blakiston, Philadelphia.

⁴ Cloudman, A. M., *Biol. Lab. Mouse*, 1941, 168, Blakiston, Philadelphia.

⁵ Emmel, V. E., Weatherford, H. L., and Streicher, M. H., *Am. J. Anat.*, 1926, 38, 1.

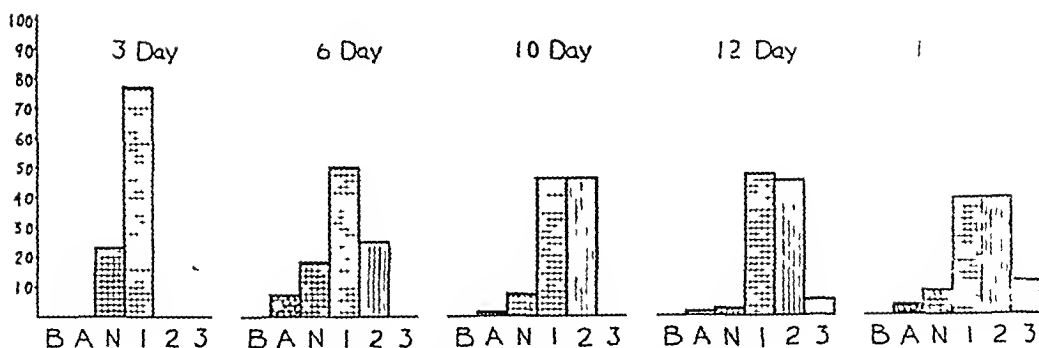


Fig 2

Polygon showing the distribution of the differential counts

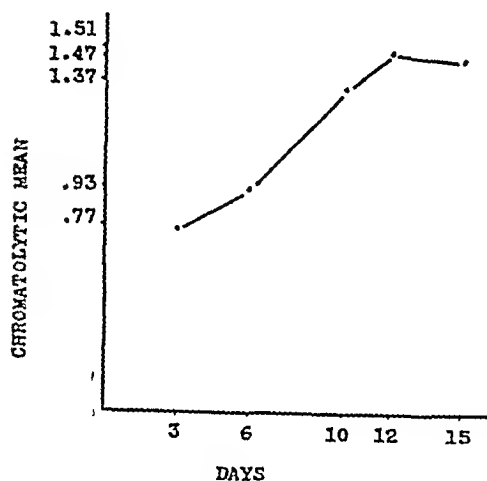


Fig 3

Graph showing the relation of the chromatolytic mean index to time

hyperchromic and the pyknotic cell

Experiment Five cats were anaesthetized with pentobarbital and, with sterile operative technic, the first sacral ventral root on the right side of each was sectioned near the spinal ganglion. The animals were allowed to survive for 3, 6, 10, 12, and 15 days. They were killed with pentobarbital and

TABLE II
Differential Counts of Motor Neurons

Lvp	Days	B	A	N	1	2	3	Mean
222	3	0	0	23	77	0	0	77
223	6	0	7	18	50	25	0	93
224	10	0	1	7	46	46	0	137
225	12	0	1	2	47	45	5	151
226	15	0	3	8	39	39	11	147

perfused with 10% formalin. The spinal cords were removed, sectioned, stained with cresyl violet and differential counts of the degenerating cells of each preparation were made according to the classification described above.

The polygons illustrate the sequence of changes which follow root section in these animals. Weighting the columns in Table II by multiplying Col B by -2 , A by -1 , N by 0, 1 by 1, etc., allows calculation of the mean for each animal. The relation of the mean to time is shown in Fig 3.

Summary and Conclusions The gradual and sometimes subtle differences in cell groups undergoing chromatolysis for varying periods of time may be detected only with a quantitative method. The index presented above serves this purpose and is yet simple and practicable.

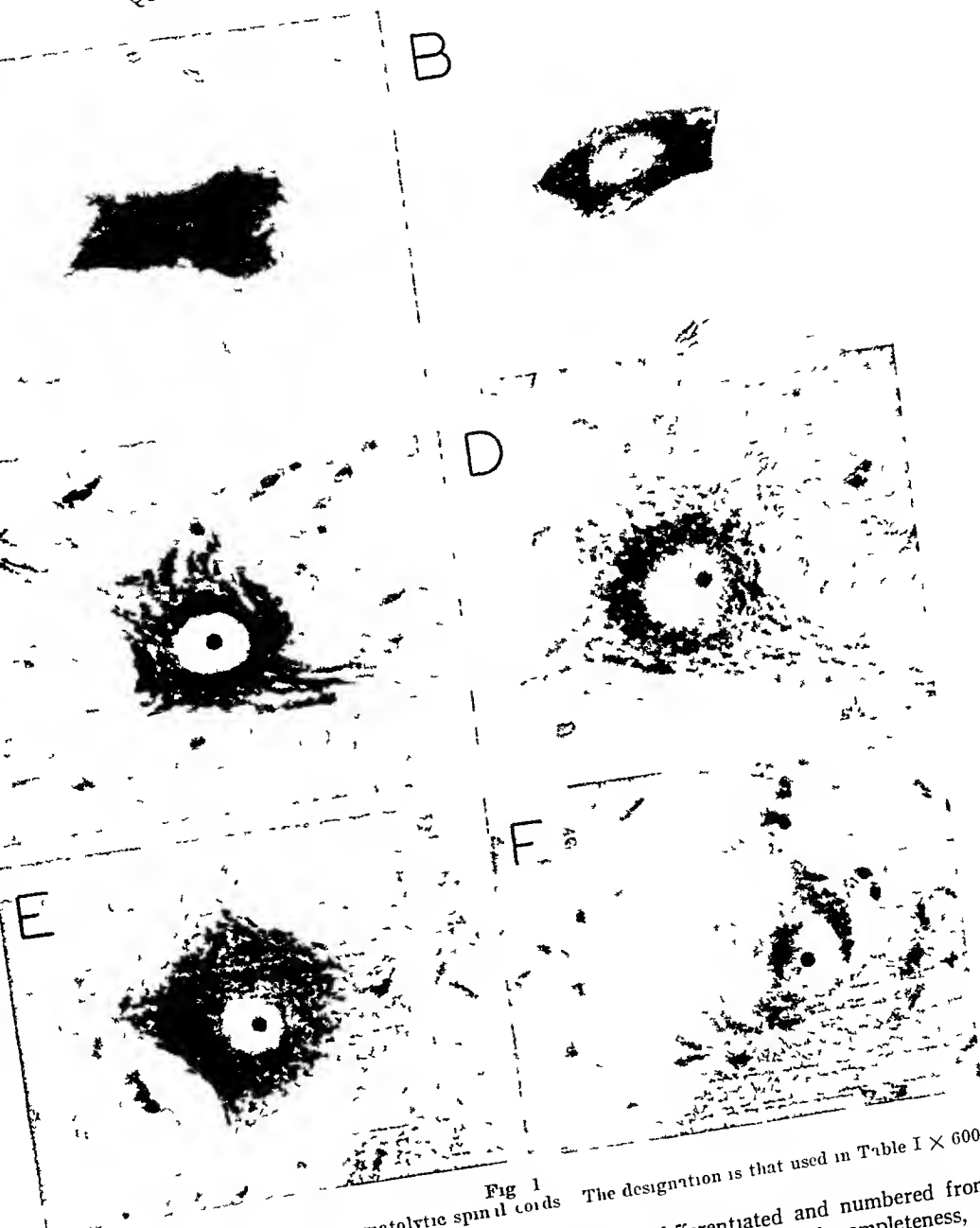


Fig 1

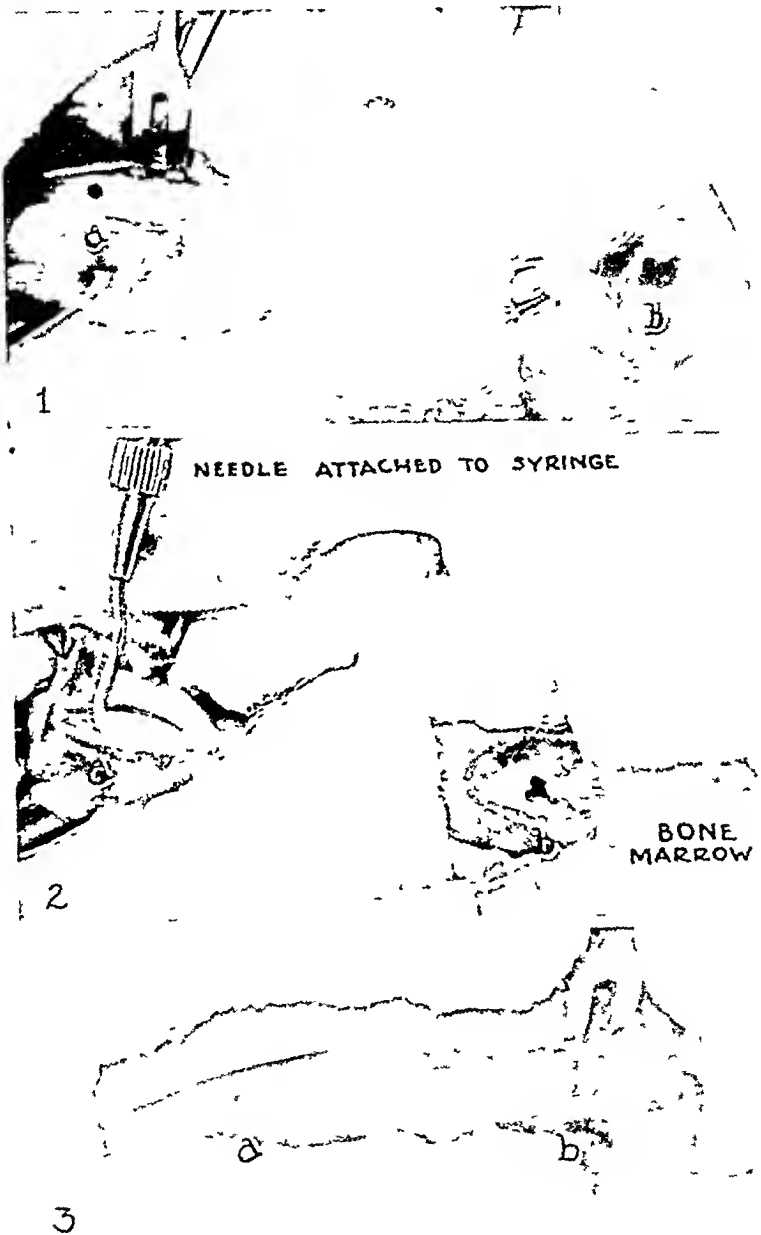
Photomicrographs of cells from chromatolytic spinal cords. The designation is that used in Table I $\times 600$

servations

In addition to the normal cell, which we have designated N in the accompanying schema, 3 stages, chosen with regard to the accuracy with which they may be identified,

have been differentiated and numbered from 1 to 3. For the sake of completeness, 2 additional categories, A and B, have been set up on the opposite side of the scheme from the chromatolyzed cells to include the

Fig 1
Steps in Removing the Lature Marrow from a Long Bone of a Living Rabbit



Two holes drilled in the femur with a Rask nail drill. At one end of the bone a single hole is made (1a). At the opposite end 4 holes are made and the central bone spicule is removed leaving a larger opening (1b). A flexible silver cannula is inserted into the single hole (2a) and with a syringe containing oil or water, the marrow is expressed through the larger opening at (2b). Both openings are then sealed with bone wax (3a and b).

Removal of Bone Marrow in Living Animals

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Experimental suppression of bone marrow activity has been achieved by means of certain chemicals such as benzene and by irradiation. No method has been available for the removal of the entire marrow. The procedure to be described allows the complete extirpation of the whole marrow from the shaft of any of the long bones of a living animal.

Method. The animal of choice is the rabbit. In rabbits along with mice, rats and guinea pigs the marrow in the shaft of the long bones is tubular with relatively few connective tissue bands which binds it to the endosteum. The rabbit is preferable because being the largest of the animals with tubular marrow, the operative procedure involved in the method is technically more accessible. The animal also lends itself readily for hematologic studies.

The animals were anesthetized with pentobarbital sodium. The hair was removed from the extremity with a depilatory preparation and wrapped with cotton saturated with a mild antiseptic. The subsequent surgical procedures were carried out under aseptic precautions to prevent infections of the bone cavity. In the removal of tibial marrow, 2 small incisions are made at each end of the bone. One over the broad anterior surface of the proximal end of the bone and the other over the distal end. A cross incision is made on the broad end to the periosteum and the soft tissue is pulled away. At the narrower end of the bone tendons are retracted. In the case of the femur, humerus, radius and ulna, a single incision from the proximal to the distal ends is preferable. The muscles are separated along the fascial lines and retracted. With a Rask nail drill a single opening is made at the narrower end through the bone into the marrow cavity (Fig 1 1a). In the wider end, 4 holes are drilled and the bone which is

outlined is removed leaving a large opening (Fig 1 1b).

A flexible silver cannula is inserted into the small opening (Fig 1 2a). The cannula must fit the opening tightly. A syringe filled with sterile mineral oil is attached to the cannula. The pressure of the oil separates the marrow and expresses it out of the bone cavity through the larger opening (Fig 1 2b). The bone cavity is flushed with warm saline. Some marrow remains in the epiphysis of the bone, partly within soft cancellous tissue. If the experiment requires the removal of the entire marrow, the cancellous tissue is first macerated with a fairly rigid metal probe with a hooked tip. The marrow is then washed out with warm saline using the silver cannula and the syringe. There may be severe bleeding from the epiphyses. The bleeding may be controlled by packing both ends with thinly rolled gauze saturated with bone wax. One end of the gauze is left out at the opening. The packing may remain for 10 to 15 minutes or in case of intractable bleeding for 24 to 48 hours at which time it is removed. The openings in the bone are sealed with bone wax (Fig 1 3a,b).

If the tissues are treated with consideration and the muscles are not torn or crushed, the animal shows no ill effects. Mistreatment such as crushing or tearing of tissue may result in shock and death. Undue pressure on the inner structures of the epiphyses predisposes the animal to shock.

The removal of the entire marrow allows the study of the mechanism of whole marrow regeneration and places of origin. The method also presents a testing procedure for the action of substances on regeneration of marrow.

Summary. A method is described allowing the removal of the entire marrow from any of the long bones in a living rabbit.

TABLE I
Perosis Caused by the Feeding of Thiouracil

Group	Supplement to normal control ration	Total No chicks started	No alive at 5 wk	No with perosis at 5 wk	Avg weight at 5 wk, g
1	No supplement	41	41	2	345
2	0.5% thiouracil	34	31	30	251
3	0.5% thiouracil, + 0.3% choline, + 10 mg nicotinic acid, + 40 μ biotin + 100 mg MnSO ₄ per 100 g of ration	10	10	10	229
4	0.5% thiouracil + 2 mg riboflavin per 100 g of ration	6	6	6	244

to prevent an inward curling of the toes was added to the thiouracil ration (group 4). Again, no improvement was noted either in perosis or in the condition of the toes.

In addition to the leg disorder, which caused some difficulty in walking, chickens fed the thiouracil ration showed certain other external symptoms. They grew at a somewhat slower rate than did the control chickens and appeared very fat and stocky. Feather growth was greatly retarded and feathers on the breast and lateral tracts which did appear were very lacy in structure as described by Juhn.¹⁴ In the New Hampshire chickens little, or none, of the usual black pigment appeared in the wing or tail feathers. Comb growth of the males was retarded to such an extent that it was extremely difficult to distinguish the males from the females, even after 6 or 8 weeks. A small percentage of the chickens receiving the thiouracil had excessively scaly feet. Efficiency of feed utilization was similar to the control group.

Autopsies of a few representative thiouracil-treated birds showed the presence of an excessive amount of fat in the abdominal cavity and throughout the body generally, including the muscle tissues. The thyroids were enlarged. These findings are in accord with those of Missouri workers.^{15, 16}

Discussion The mechanisms and reactions involved in the formation of perosis are unknown. Wiese, *et al* reported that

there was a decrease in the amount of bone phosphatase activity in chickens deficient in manganese.¹⁷ This was not found to be the case in a choline deficiency.⁴ It is probably true that a variety of reactions are involved as evidenced by the number of nutrients necessary to prevent this disorder.

Although no direct evidence is given, the work presented in this study suggests that a lack of thyroxine may also be involved in perosis since it is well accepted that thiouracil depresses the formation of thyroxine (see reviews by Juhn¹⁴ and the Missouri workers).^{15, 16} If this suggestion is true, perosis should be obtained either by thyroidectomy or by feeding a diet deficient in iodine to chickens. Perosis apparently has not been noted under these conditions.

In this connection, and since a manganese deficiency in the chicken results in perosis, the observation of Reineke and Turner is interesting,¹⁸ that manganese may promote the formation of thyroxine, *in vitro*.

The perosis obtained in this study may be developed independently of the thyroid gland. Thiouracil has been reported to have a direct effect on certain enzymes such as tyrosinase,¹⁹ and on the respiration of bone marrow cells,²⁰ in addition to its effect on cytochrome oxidase of thyroid tissue.²¹

¹⁷ Wiese, A. C., Benham, G. H., Elvehjem, C. A., and Hart, E. B., *Poultry Sci.*, 1941, **20**, 255.

¹⁸ Reineke, E. P., and Turner, C. W., *J. Biol. Chem.*, 1945, **161**, 613.

¹⁹ Prischke, K. E., Cantarow, A., Hart, W. M., and Rakoff, A. E., *Proc. Soc. Exp. Biol. and Med.*, 1944, **57**, 37.

²⁰ Warren, C. O., *Science*, 1945, **102**, 175.

¹⁴ Juhn, M., *Endocrinology*, 1944, **35**, 278.

¹⁵ Mixer, J. P., Reineke, E. P., and Turner, C. W., *Endocrinology*, 1944, **34**, 168.

¹⁶ Schultze, A. B., and Turner, C. W., *Missouri Research Bul.*, 1945, **392**.

Perosis Caused by Feeding High Levels of Thiouracil *

GEORGE M. BRIGGS AND ROBERT J. LILLIE (Introduced by Mary Juhn)

From the Department of Poultry Husbandry, University of Maryland, College Park, Md

Perosis, or "slipped tendon," is a common deformity of the leg bones of growing chickens, and may be produced by a variety of conditions usually dietary in origin (see review by Ewing)¹ Dietary deficiencies of manganese,² choline,³ biotin,⁶ nicotinic acid,⁷ and probably unidentified factors have been known to produce this abnormality. The condition has been said to be caused also by such factors as excessive minerals in the diet,⁸ confinement, wire floors, or it may be inherited.¹⁰

In studies on the effects of thiouracil upon feather pigmentation, it was noted that this drug, when fed at a relatively high level in normal diets, caused the production of perosis in young growing chicks. Results of this and further investigations are presented herein.

Experimental Procedure and Results Day-old New Hampshire and Barred Rock \times New Hampshire crossbred chicks were used. They were raised in electrically-heated batteries with screen bottoms and given the experi-

mental ration and water *ad libitum*. The experiments were conducted for a period of 5 weeks. The thiouracil, which was added to a normal control ration (a typical poultry starting mash),¹⁰ was fed during the entire period.

As may be seen from the summary of results presented in Table I, the addition of 0.5% of thiouracil to the normal control ration resulted in a very high incidence of perosis by the end of the fifth week. The condition was very similar to that ascribed to high levels of minerals in the diet, and to other causes, by the early workers on this problem.^{9, 11, 12} and was usually noticeable by the end of the fourth week. The first external symptom noted was a swelling of the tibia-metatarsal joint followed by a shortened appearance and bending of the metatarsus. This was usually accompanied by an outward curling of the toes which has been noted previously in perosis and distinguished from the curled-toe condition of nutritional paralysis.¹³ In only about 20% of the chickens was there an actual displacement of the tendon from its condyle.

One group of 10 chicks (group 3) was given, in addition to the thiouracil, a mixture of nutrients known to be necessary to prevent perosis under normal conditions. This was done in an attempt to counteract the effects of thiouracil, if possible. The mixture included choline, nicotinic acid, biotin, and manganese at levels well exceeding ordinary requirements. No improvement was noted.

Because many of the chickens had an outward curling of the toes, riboflavin, known

* Scientific paper No. A 126. Contribution No. 2014 of the Maryland Agricultural Experiment Station.

¹ Ewing, W. R., *Handbook of Poultry Nutrition*, 1943, chap. 11, 403.

² Wilgus, H. S., Jr., Norris, L. C., and Heuser, G. F., *J. Nutrit.*, 1937, **14**, 155.

³ Jukes, T. H., *J. Nutrit.*, 1940, **20**, 445.

⁴ Hegsted, D. M., Mills, R. C., Elvehjem, C. A., and Hart, E. B., *J. Biol. Chem.*, 1941, **138**, 459.

⁵ Hogan, A. G., Richardson, L. R., Patrick, H., and Kempster, H. L., *J. Nutrit.*, 1941, **21**, 327.

⁶ Jukes, T. H., and Bird, F. H., *Proc. Soc. Exp. Biol. and Med.*, 1942, **49**, 231.

⁷ Briggs, G. M., Jr., Luckey, T. D., Teply, L. J., Elvehjem, C. A., and Hart, E. B., *J. Biol. Chem.*, 1943, **148**, 517.

⁸ Hunter, J. E., and Funk, E. M., *Proc. 22nd Poultry Sci. Assn.*, 1930, p. 45.

⁹ Serfontein, P. J., and Payne, L. F., *Poultry Sci.*, 1934, **13**, 61.

¹⁰ Briggs, G. M., and Jull, M. A., *Maryland Ext. Bul.*, 1946, **109**.

¹¹ Herner, M. C., and Robinson, A. D., *Poultry Sci.*, 1932, **11**, 283.

¹² Titus, H. W., *Poultry Sci.*, 1932, **11**, 117.

¹³ Norris, L. C., Heuser, G. F., Wilgus, H. S., Jr., and Ringrose, A. T., *Poultry Sci.*, 1931, **10**, 93.

TABLE I
Cross Reactions Between 188 Serums Which Gave at Least One 2 Plus (++) or Stronger Precipitate

Nature of serums used		No of tests	Reaction ++ or more	Reaction + to '2	Reaction 0
Serum 1	Serum 2				
A	B	438	237	114	89
A	AB	69	43	17	9
B	AB	77	20	12	16
A	A	148	1	3	144
B	B	88	0	20	59
AB	AB	3	1	2	0
Totals		803	309	177	317

should be reversed if antigen or antibody is actually implicated)

Methods We have found that a study of these phase substances was facilitated by bleeding several patients during the first 3 days of acute rheumatic fever and other patients in the 10-20th day of the disease and testing all serums so obtained against each other. The serums fell into 4 groups (1) A, (2) B, (3) A and B mixed, (4) negative. Further serum specimens were then classified by testing against one or more A serums and against one or more B serums.

Blood serum was preserved with merthiolate in a final concentration of approximately 1-10,000 and stored at 5°C. Centrifugation prior to use was sometimes necessary to clear. About 0.1 cc of A serum was mixed by shaking with 0.1 cc B serum in a chemically clean precipitin tube, which had an inner diameter of 2 mm, incubated in a water bath at 41°C 2 hours, and the readings made with the aid of a dualoupe binocular 3 X magnifier. The tubes were then placed in the refrigerator overnight, allowed to warm to room temperature and read again. The serums should not be allowed to freeze. The suspended precipitate after overnight refrigeration was sometimes stronger, but more anomalous effects appeared than if the reading was made on a basis of the results after 2 hours at 41°C. The observed reaction was in the form of a suspended precipitate which looked much like a positive Kahn test. The intensity was read as +++ flakes, ++ large granules, + small granules, ± very fine granules, 0 clear. Miss Pauli viewed some of the readings and found them to cor-

respond with those reported in 1939. It is important that every serum be set up by itself as a control because some serums precipitate spontaneously. These were thought to contain a mixture of A substance and B substance. Such serums often contained predominantly one or the other. As a serum became over 2 months old there was an increasing tendency for it to precipitate by itself or for it to lose potency even though it did not become self-precipitating.

Practically all tests were with heterologous serums. Only a small series was with homologous serums, sufficient to confirm the fact that A serum would react with B serum from the same patient.

The most difficult aspect was to secure and maintain a supply of serums containing dependable A and B substances. In civilian life this is accentuated by the small amounts of blood available from children. No sooner is a serum well established as having a dependable A or B content which could be used to classify other serums than it is exhausted.

Cross Reactions Between Serums Table I summarizes the results of 803 tests on 188 serums from 123 patients. Of the 188 serums, 115 were predominantly A serums, 59 were B serums, and 14 were mixed. Of the 123 patients, 51 had rheumatic fever, 40 atypical pneumonia, 23 nasopharyngitis, and 9 miscellaneous conditions, including 2 with mumps.

This table includes all the serums which gave at least one 2 plus reaction. Only the maximum reaction between 2 serums is recorded in case 2 serums were tested against one another more than once.

It should be stressed that the level of thiouracil used in this study is higher than that used by most investigations with this drug

Summary Perosis, as characterized by an enlargement of the tibia-metatarsal joint and by a thickening and bending of the metatarsal, was obtained in young chicks by the feeding of relatively high levels of thiouracil (0.5%)

† Pischelis, K. L., Cintonio, A., and Tillson, L. K. *Proc Soc Exp Biol and Med* 1945, 60, 148

over a period of 5 weeks. The disorder was not prevented by the addition of manganese, choline, nicotinic acid, biotin, or riboflavin to the thiouracil-containing ration. It is suggested that thyroxine may in some manner aid in the prevention of perosis under normal conditions, although no direct proof for this postulation is offered.

The authors are indebted to Merck and Co., Inc., Rahway, N. J., for the crystalline vitamins, and to Lederle Laboratories, Inc., New York, N. Y., for the thiouracil.

15344

Serum Precipitation Reaction in Rheumatic Fever and in Other Conditions

A. G. WEDUM[†] AND BERNICE G. WEDUM

From the Departments of Bacteriology and Medicine, University of Colorado, School of Medicine

Coburn and Pauli¹ described a precipitation reaction which appeared when the serum of a patient in the pre-rheumatic (post-streptococcal) state or Phase II was mixed with the serum of a patient in the acute stage of rheumatic fever or Phase III. The serum in the post-streptococcal period was considered to contain an "antigen" and the serum in the acute stage of rheumatic fever to contain an "antibody." Convalescent serum also contained "antigen." No report confirming or failing to confirm this work has appeared in print. Personal conversation has established, however, that these experiments have been repeated by several workers who have seen the precipitation described, but who could not ascribe any significance to it because of the irregularity of its occurrence.

During 1944-45 through the courtesy and

interest of the medical officers* in the Army Air Force stations at Lowry Field, Buckley Field, and Fort Logan, the authors were able to study the serums of some 491 men with diagnoses as follows: 122 rheumatic fever, 181 nasopharyngitis, tonsillitis, and sinusitis, 38 atypical pneumonia, and 150 miscellaneous. The purpose of this paper is to indicate the scope and limitations of the serum precipitation reactions as observed and to suggest a possible relationship to the occasional unfavorable response of patients with acute rheumatic fever to transfusion with blood or plasma.

Terminology Because of the unknown nature of the substances involved in the reaction, it has seemed desirable to avoid the designations "antigen" and "antibody." Yet to facilitate comparison with the report of Coburn and Pauli, the term "A substance" is used to refer to the "antigen" reactive substance present in the serum prior to the onset of acute rheumatism or during convalescence or a remission, and the term "B substance" is used to refer to the "antibody" reactive substance present during active rheumatism. (It is likely that these terms

[†] Present address: Camp Detrick, Frederick, Md.
1 Coburn, A. F., and Pauli, Ruth H., *J. Exp. Med.*, 1939, 69, 143.

* Especially Lt. Col. L. D. Williams, Capt. C. L. Hoff, Capt. G. L. Erdman, Major O. G. Wilson, Lt. Col. W. B. Admson, Capt. G. Blankfort, Major F. Foster, Major L. R. Grims.

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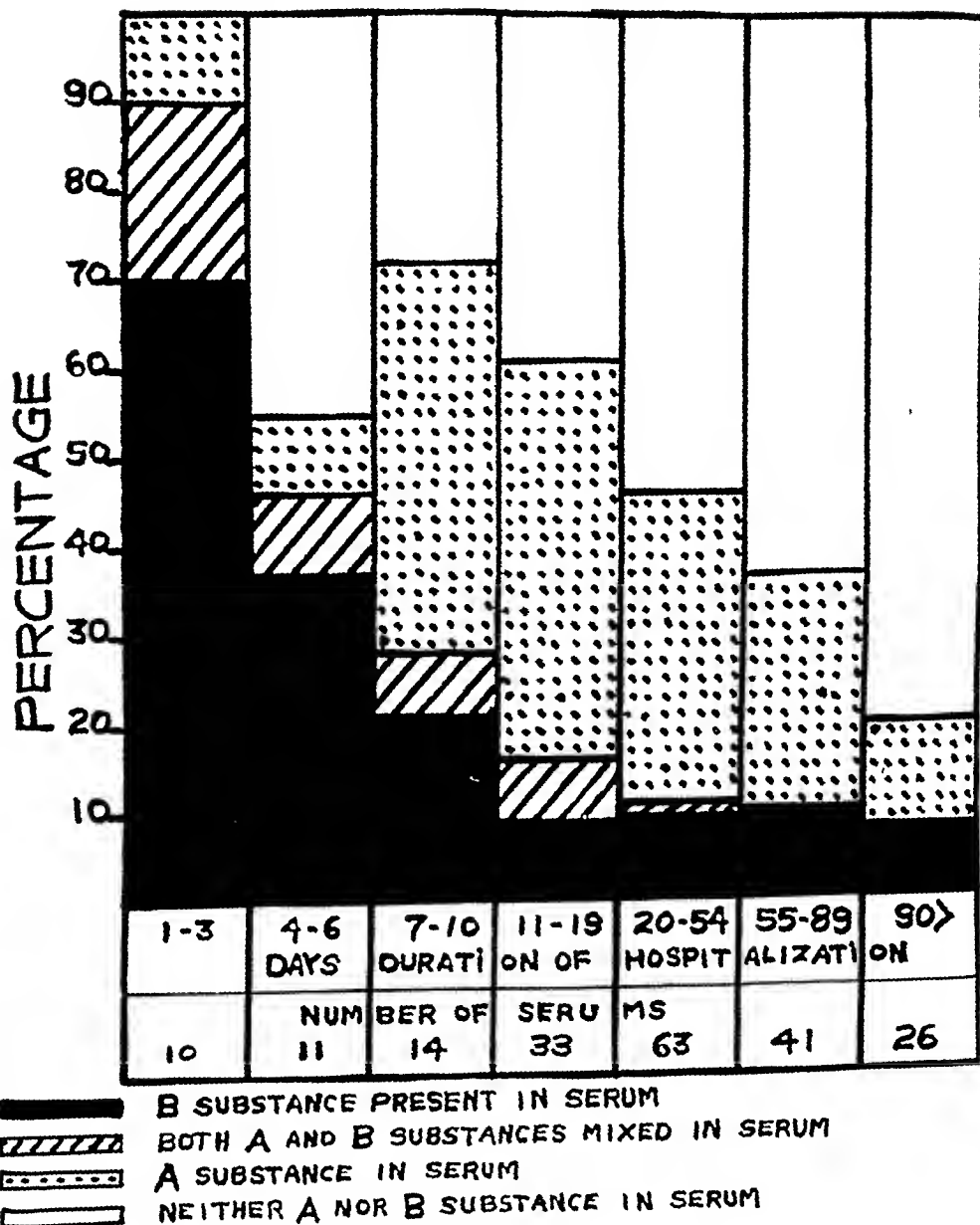


Fig 1

Distribution of A and B substances in 98 patients with rheumatic fever in relation to duration of hospitalization

Table I shows that in 438 tests of an A serum against a B serum there were 2 plus reactions in 235 tests and no reactions in 89 tests, which suggests a quantitative (possibly also qualitative) difference in amount of A or B substance present in any serum. When

88 tests were made by combining one B serum with another, there were 29 weak reactions and 59 negatives. This suggests that the B serums had only a predominant content of this substance but that they did contain some small amount of A substance also.

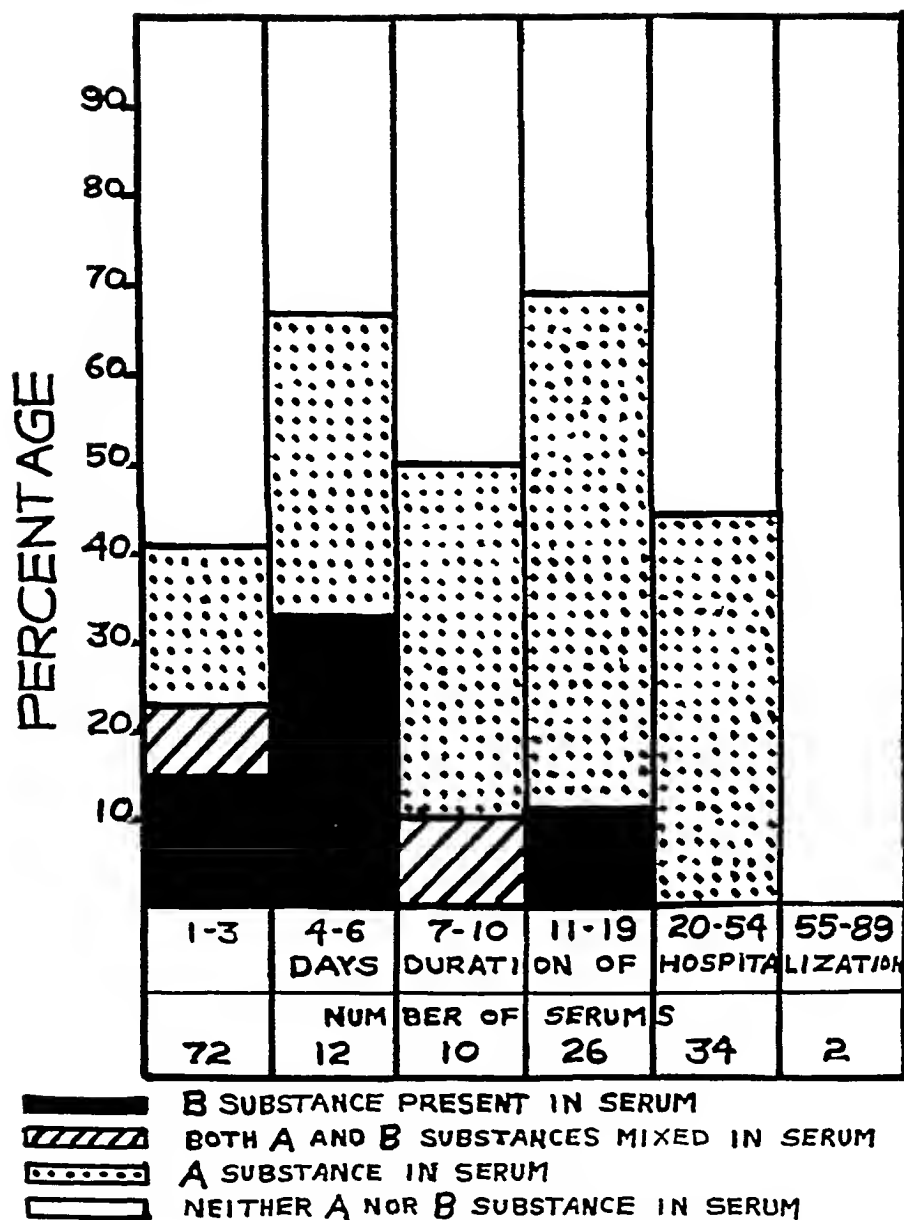


Fig. 2

Distribution of A and B substances in 116 patients with nasopharyngitis in relation to duration of hospitalization

Serums from patients with rheumatic fever, atypical pneumonia, and nasopharyngitis cross-reacted, hence the phenomenon was not exclusively connected with any specific disease

A and B Substances in Relation to Duration

of Disease Fig 1 presents the results obtained with 198 serums from 98 patients with rheumatic fever, diagramed to show whether the patients were in an A or B phase at the time the blood was drawn. No patient appears in any one category more than once

The first day of disease was considered to be the first day of hospitalization. This is a reasonably accurate assumption for the military men concerned because hospitalization usually occurred early.

It can be seen that during the first 3 days of hospitalization 7 of 10 patients were in the B phase but that after the 10th day only 15 of 163 sera contained this substance predominantly. Those which persisted in a B phase after the 40th day were principally instances of recurrences of rheumatic fever or prolonged active rheumatic fever. The number in the A phase was low at first, increased until about the 20th day, and then declined while those with negative serums increased. This same general tendency was also present in 87 serums from 34 patients with atypical pneumonia and in 156 serums from 116 patients with nasopharyngitis, but in both instances there were more serums which contained neither A nor B substance. This is illustrated for nasopharyngitis in Fig 2. Serums from patients with rheumatic fever contained A or B substances more frequently than did those from other conditions studied by us.

Sequence in the Appearance of B and A Substances There was a definite order of appearance of B and A as follows: (1) acute illness-B phase, (2) subsidence of illness-mixed B and A, (3) convalescence-A phase, (4) recovery-negative phase, (5) recurrence of illness-B phase. This is illustrated in Fig 3 which shows 85 serums from 28 patients whose diagnoses were as follows: 14 rheumatic fever, 7 nasopharyngitis, 5 atypical pneumonia, and 2 nasopharyngitis with later rheumatic fever. Multiple specimens of serum were taken from each patient. The time intervals varied, but each patient was in the B phase when first bled. Presence of B phase in the 4th, 5th and 6th bleedings was found in 2 patients with prolonged active rheumatic fever. Evidence of the above sequence was also seen in 10 patients whose first serum contained mixed A and B, and in 32 who were in the A phase when first bled. In 41 patients initially negative, a number went into the A phase without passing through an observed B phase.

In all these observations consideration must be given to the variable titer of the serums and the irregularity of the times at which patients were bled, which caused difficulty in classification of serums and detection of the sequence of phase substances.

The following case histories will illustrate the sequence of events.

Hospital day	Case
1	Scarlet fever
14	Acute R F with polyarthritis
61	O phase substance in serum
77	Recurrence of acute R F
81	Only B substance in serum
93	Only A substance in serum
123	O phase substance in serum
124	Returned to active duty
Case 26	
1	Atypical pneumonia
3	X-ray L L L atypical pneumonia
12	X-ray resolved atypical pneumonia
14	Only A substance in serum
18	X-ray recurrence atypical pneumonia
20	Only B substance in serum
26	X-ray resolved atypical pneumonia
41	Only A substance in serum
57	Returned to active duty
84	O phase substance in serum (on duty), no prior history of R F, no arthritis or carditis during hospitalization
Case 62	
1	Acute R F with polyarthritis
4	Only B substance in serum
10	Only B substance in serum
36	Only A substance in serum
66	Only A substance in serum
97	O phase substance in serum
100	Sent to Arizona as convalescent R F
Case 123	
1	Nasopharyngitis
2	Mixed A and B substances in serum
4	Only A substance in serum
7	Returned to active duty
48	O phase substance (on duty)

Phase Substance in Relation to Transfusions In patients with rheumatic fever partial collapse has been reported after the administration of convalescent serum² and recurrence of rheumatic fever after administration of serum obtained during the acute phase of the disease in the donor.³ Blood plasma from normal donors caused an increase in rheumatic activity in 7 of 9 patients.⁴ At

² Green, C. A., Glazebrook, A. J., Thompson, S., and Hopkins, W. A., *Proc Roy Soc Med*, 1940, **33**, 275.

³ Friedman, M., Klein, R., and Rosenblum, P.,

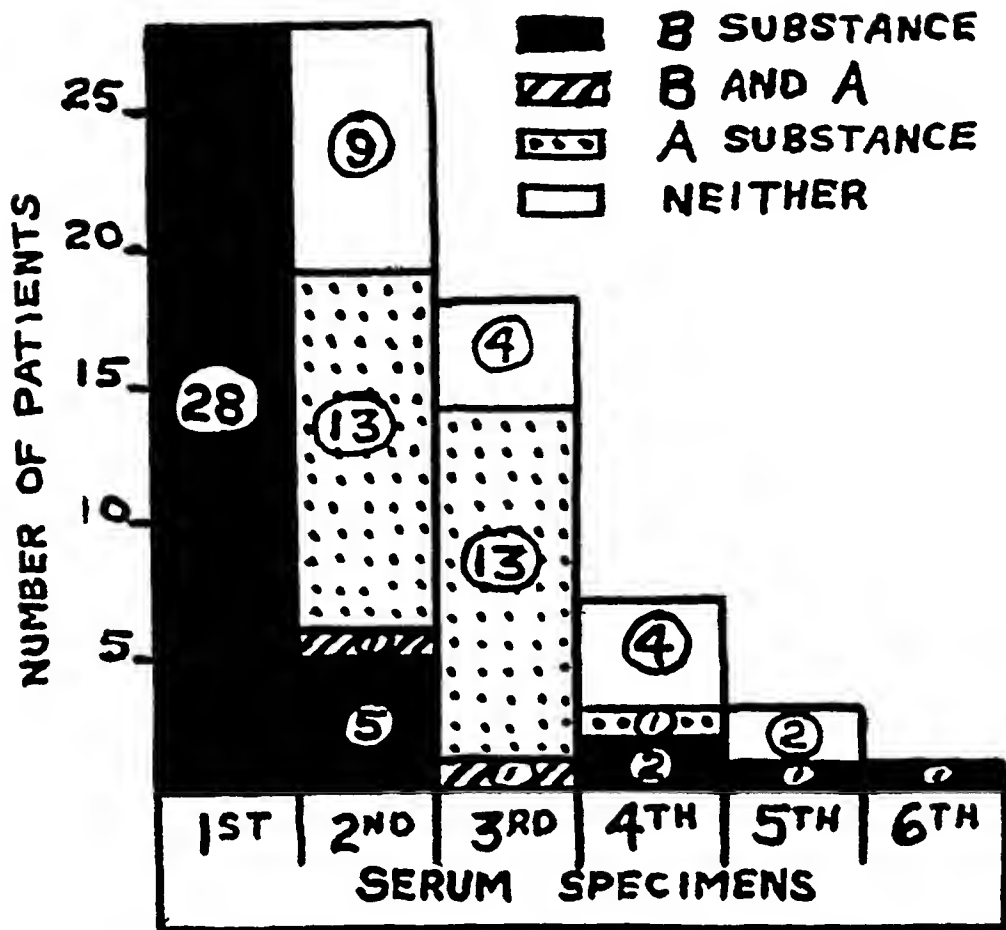


Fig 3

Sequence of B and A substances in 28 patients yielding multiple serum specimens at irregular time intervals during and after hospitalization

Lowry Field, 2 patients had an unfavorable clinical response to compatible blood transfusions. It may be significant that in each case the recipients were definitely in B phase and the 5 donors were in an A phase, so that when the serums of recipient and donor were mixed, a suspended precipitate formed. Among 64 recipients of transfusions at Colorado General Hospital, the B substance was present in 16 patients, in such diverse conditions as Hodgkins disease, active tuber-

culosis with abortion, lung abscess with surgical drainage, carcinoma of the stomach, burns, abscess posterior to the stomach from perforated peptic ulcer, arteriosclerotic gangrene with amputation. Among 38 civilian donors there was a questionable A phase in only 2 and no B phase. Study of this "phase reaction" in a large transfusion service might yield interesting results.

Other Experiments In a few trials, heating serums to 55°C for 30 minutes decreased their ability to react. Complement had no restorative effect. In one serum use of a fritted glass filter quantitatively reduced the potency of the A substance, but Berkefeld filtration did not appreciably affect either A

Am J Dis Child, 1938, 50, 1304

⁴ Griffith, G. C., Leike, W. H., and Butt, Hugh, *Modern Concepts of Cardiovascular Disease*, published monthly by the American Heart Assn 1945, 14, 7

or B substances in 3 instances. No absolute correlation was observed between presence or absence of A or B substances and the blood group of the donors,[†] blood sedimentation rate,[†] serum antistreptolysin titer,[†] warm or cold agglutinins for red blood cells, sheep red cell heterophile antibody titer,[†] or the presence of hemolytic streptococci in the throat.[†]

Comment. A clear serum tended to precipitate with a turbid (even after centrifugation or filtration) serum but there was no uniform relationship. Two clear serums or 2 turbid serums might react. Possibly the turbid serum merely furnished a suitable colloidal state similar to that when colloidion particles are used to detect small quantities of antibody.

Whether the reactions observed are true immunological phenomena or only aspects of serum lability is not clear. The similarity to the precipitinogen in yellow fever as described by Hughes⁵ and Lennette⁶ is noteworthy. There have been reports which suggest the existence of similar reactive substances in typhus⁷ post-vaccinal hepatitis,⁸ and infectious myxomatosis of rabbits.⁹ There may exist a whole series of closely related auto-antigens which vary slightly with each disease.

Close examination of a recent paper¹⁰ on

electrophoretic changes in the serum protein patterns of patients with scarlet fever and rheumatic fever reveals that the maximum reduction (value 0.7) in the albumin-globulin ratio in the 3 patients with rheumatic fever occurred at the onset of joint pains. This is the point at which we would be most likely to find "B substance" in the serum. Similarly the only patient with scarlet fever who developed a similar reduction of the albumin-globulin ratio was one whose course was complicated by purulent sinusitis. He also would, in our experience, be more likely to have "B substance" than those patients with uncomplicated scarlet fever. The question is then whether the B phase represents a serum lability caused by the lowered albumin-globulin ratio.

Summary. 1 When the serum of a patient in the early acute stage of rheumatic fever was mixed and incubated with a convalescent serum taken at an appropriate time from himself or another rheumatic patient, a precipitate formed. 2 The "phase substances" causing this reaction were present, but less frequently, in atypical pneumonia, nasopharyngitis, several other diseases, and rarely, in apparently normal blood donors. 3 Because cross-reactions occurred between serums from patients with different diseases, it is difficult to ascribe any etiological significance to the findings. 4 This reaction may be responsible for the occasional unfavorable response to blood or plasma transfusion of patients with rheumatic fever. 5 It is not clear whether this phenomenon is a manifestation of serum lability or of the formation of an auto-antigen-antibody system.

The authors wish to thank Miss Ella Jane Swan for her voluntary technical assistance and Dr Homer F. Swift for his criticism and comments.

[†] These procedures were performed by the A. A. F. Laboratories at Lowry and Buckley Fields.

⁵ Hughes, T. P., *J. Immunol.*, 1933, **25**, 275.

⁶ Lennette, E. H., and Perlowagora, Alina, *Am. J. Trop. Med.*, 1945, **25**, 11.

⁷ Smorodintzeff, A. A., and Fradkina, R. V., *Proc. Soc. Exp. Biol. and Med.*, 1944, **50**, 93.

⁸ Sawyer, W. A., Meyer, K. F., Eaton, M. D., Biucr, J. H., Putman, P., and Schwentker, F. F., *Am. J. Hyg.*, 1944, **40**, 35.

⁹ Rivers, T. M., Ward, S. M., and Smadel, J. E., *J. Exp. Med.*, 1939, **69**, 31.

¹⁰ Dole, V. P., Watson, R. F., Rothbard, S.,

Braun, E., and Winfield, K., *J. Clin. Invest.*, 1945, **24**, 648.

The Antagonistic Action of Choline and its Triethyl Analogue

ALBERT S. KESTON AND S. BERNARD WORTIS (Introduced by Isidor Greenwald)

From the Biochemical Laboratories, Department of Psychiatry, New York University

In recent years, considerable interest has been developed in the interference with biological processes through the use of analogues of essential metabolites.

Triethyl choline chloride, the ethyl analogue of choline, is an interesting compound from this point of view. Triethyl choline is not capable of replacing choline in biological processes in which choline acts as a methyl donor,¹ but its lipotropic activity has been found to be substantially equal to that of choline itself.² Since the formation of acetyl choline presumably would not involve transmethylation directly, the triethyl derivative might possibly be handled similarly to choline in the systems involving its acetylation. The acetyl derivative of triethyl choline, however, does not have any of the stimulating actions of acetyl choline. A competition with choline in these systems should therefore lead to diminished formation of active acetyl derivatives.

Triethyl choline chloride, when injected into animals in sufficient amounts, is acutely toxic.³ The symptoms demonstrated include apparent muscular weakness, apparent exophthalmos, and convulsions, followed by death within a few minutes. On the assumption that this acute toxicity is due to competition with choline in an essential biological system, choline was administered to see whether it would protect animals against its triethyl analogue.

Forty-four mice, ranging in weight from 16 to 23 g, were divided into 2 groups containing animals of corresponding weights.

* Aided by a grant from the John and Mary Markle Foundation.

¹ Moyer and du Vigneaud, *J. Biol. Chem.*, 1942, **143**, 373.

² Channon, Platt, and Smith, *Biochem. J.*, 1937, **31**, 1736.

³ Hunt and Tiveru, *J. Pharm. and Exp. Therap.*, 1909 **10**, 1, 303.

The mice of one group were injected in the hind leg with triethyl choline chloride, prepared according to the directions of Channon and Smith.⁴ Six out of 9 mice given 125 mg died. Twelve out of 13 mice given 175 mg died.

The mice of the other group were treated with the same amounts of triethyl choline chloride and an equal weight of choline chloride. Out of 9 mice given 125 mg triethyl choline chloride together with 125 mg choline chloride, all survived. Out of 13 mice given 175 mg triethyl choline chloride together with 175 mg choline chloride, 12 survived.

In order to investigate further the mechanism of the toxicity of the compound, the contraction of isolated frog muscles (gastrocnemius and rectus abdominus) was studied in the presence and absence of the triethyl derivative. The muscle was suspended from a muscle lever in the usual way in frog Ringers solution and the contractions recorded with a kymograph. When triethyl choline was placed in the solution bathing the muscle, it very substantially blocked the response of the muscle to choline, while it did not appreciably block the response to acetyl choline. On washing the muscle, the response to choline was very largely restored. When muscles were contracted with choline, addition of the triethyl derivative to the fluid containing the choline, resulted in a rapid relaxation of the muscles. No such effect was observed when acetyl choline was employed. Table I is a summary of a typical experiment.

These findings are consistent with the assumption that the compound interferes with acetyl choline formation if choline does not act directly on nerve-muscle preparations but acts by forming or making available acetyl choline. Aside from the fact that choline

⁴ Channon and Smith, *Biochem. J.*, 1936, **30**, 115.

TABLE I

Muscle <i>Protoplastococcus</i> Liver num 1 12				
Time	Substance added	Concentration mg per ml	Contraction in mm	
1 24	acetyl choline chloride	0.015	28	
28	wash 3 times			
30	acetyl choline chloride	0.015	28	
34	wash 3 times			
45	choline chloride	1.3	26	
50	wash 3 times			
2 00	triethyl choline chloride	1.3	0	
15	choline chloride	1.3	3	
20	wash 3 times			
23	choline chloride	1.3	19	
31	wash 3 times			
58	acetyl choline chloride	0.015	29	
3 01	wash 3 times			
20	acetyl choline chloride	0.015	27	
24	wash 3 times			
26	triethyl choline chloride	1.3	0	
40	acetyl choline chloride	0.015	29	

is a presumable precursor of acetyl choline, choline may act to liberate acetyl choline by cationic exchange adsorption, as suggested for the mode of action of choline derivatives by Renshaw, Ziff and Green⁵

⁵ Renshaw, Ziff, and Green, *J Pharm and Therap*, 1938 **62**, 430

An interesting aspect of triethyl choline chloride is that it represents a compound which can replace a metabolite in one process but is antagonistic to it in another physiological function

The authors are indebted to Miss Edith Ludmer for her assistance in these experiments

15346 P

Anticoagulant Activity of the Trypsin Inhibitor from Soya Bean Flour

HENRY J. TAGNON AND JEAN P. SOULIER (Introduced by F. H. L. Taylor)

From the Thorndike Memorial Laboratory Boston City Hospital, and the Department of Medicine, Harvard Medical School Boston

We have observed that the trypsin inhibitor from unheated soya bean flour^{1,2} has marked anticoagulant activity on human whole blood, recalcified plasma and recalcified plasma to which an excess of thromboplastin is added (Table I). However, the soya bean inhibitor has no antithrombic activity. We have also observed that this material inhibits the proteolytic enzyme of blood plasma,³ which may play a role in the co-

agulation of blood. This is true whether the plasma enzyme has been activated by chloroform treatment or by the addition of streptolysin^{4,5}

A crude preparation of inhibitor was obtained by the method of Ham *et al*.¹ An excess of potassium oxalate was added to the acetone free material in order to precipitate the calcium. The solution was dialyzed 48 hours against running tap water. It was rendered isotonic by addition of NaCl and

¹ Ham, W. L., Sundstedt, R. M., and Musschl, F. E., *J Biol Chem*, 1945, **161**, 635

² Knuttz, M., *J Gen Physiol*, 1946, **29**, 149

³ Tignon, H. J., Davidson, C. S., and Taylor, F. H. L., *J Clin Invest*, 1942, **21**, 525

⁴ Christensen, L. R., *J Bacteriolog*, 1944 **47**, 471

⁵ Kaplan, M. H., *Proc Soc Exp Biol and Med*, 1944 **57**, 40

TABLE I
Anticoagulant Activity of Trypsin Inhibitor from Soy Bean Flour

Reagents							
Crystalline inhibitor (mg)	Whole blood (cc)	Citrated plasma (cc)	CaCl_2 (0.25%) (cc)	Fibrinogen (cc)	Thromboplastin (cc)	Thrombin (cc)	Clotting time
*0	2						8 min
0.2	2						18 "
†0		0.1	0.15				6 "
†0.3		0.1	0.15				21 "
0		0.1	0.15		0.1		13 sec
0.1		0.1	0.15		0.1		20 "
†0				0.1		0.1	10 "
†0.5				0.1		0.1	10 "

* 0.2 cc of saline added

† Total volume of 1 cc in test tube, by addition of imidazole buffer at pH 7.4

brought to pH 7.4 by NaOH N/10. The results obtained with the crude material were confirmed with a purified preparation 4 times recrystallized,² secured through the courtesy of Dr. Moses Kunitz.

All experiments were conducted at pH 7.2-7.4 on human blood, citrated plasma and fibrinogen, in imidazole buffer, at 37°C. The streptolysin was a dry powder, dissolved in saline.* Control experiments showed that the anticoagulant and antiproteolytic activity

were not due to the small amount of salt present in the crystalline preparation and that the addition of an excess of calcium did not modify the anticoagulant activity. Autoclaving of the crude material at 120°C for 20 minutes completely abolished the anticoagulant activity and almost completely the antiproteolytic activity.

These facts represent a new instance of the inhibition of the clotting of blood by trypsin inhibitors.^{6,7}

* Supplied by the Commission on Acute Respiratory Diseases, through the courtesy of M. H. Kaplan.

¹ Grob, D. *J. Gen. Physiol.* 1943, 26, 423.

² Ferguson, I. H. *Proc. Soc. Exp. Biol. and Med.*, 1942, 51, 373.

15347

Protective Effect of Glutathione and Cysteine Against Alloxan Diabetes in the Rat*

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From the Department of Anatomy, Western Reserve University School of Medicine, Cleveland, Ohio

Although Wiener¹ reported that alloxan produces fatal convulsions in rabbits, the mechanism of death was not understood until Jacobs² demonstrated that injections of al-

loxan into rabbits resulted in fatal hypoglycemia. It only became apparent that alloxan could produce experimental diabetes when Dunn, Sheehan, and McLetchie³ reported that alloxan produces necrosis of the

* Presented, in part, at a sectional meeting of the Proc. Soc. Exp. Biol. and Med., Cleveland, Ohio, April 13, 1945.

¹ Wiener, H., *Arch. f. exp. Path. u. Pharm.*, 1930, 42, 375.

² Jacobs, H. R., *Proc. Soc. Exp. Biol. and Med.*, 1937, 37, 404.

³ Dunn, J. S., Sheehan, H. L., and McLetchie, N. G. B., *Lancet*, 1943, 1, 484.

TABLE I

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⁵ Renshaw, Ziff, and Green, *J. Pharm. and Exp. Therap.*, 1938, **62**, 430

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15346 P

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¹ Ham, W. E., Sundstedt, R. M., and Muschel, F. E., *J. Biol. Chem.* 1945, **161**, 635

² Kunitz, M., *J. Gen. Physiol.*, 1946, **29**, 149

³ Tagnon, H. J., Davidson, C. S., and Taylor, F. H. L., *J. Clin. Invest.*, 1942, **21**, 525

⁴ Christensen, L. R., *J. Bacteriology*, 1944, **47**, 471

⁵ Kaplan, M. H., *Proc. Soc. Exp. Biol. and Med.* 1944, **57**, 40

TABLE I

Protection Against Alloxan Diabetes Protective Agent Given Intravenously 12 Minutes Before Alloxan Alloxan 40 mg/kg Intravenously, Given Immediately After Protective Agent

Protective Agent	Dose in mM/kg	No rats	% diabetic	Avg 48 hour blood sugar	
				Diabetic	Non diabetic
None	—	19	95	397	—
Cysteine	7.5	13	0	—	130
"	5.0	2	0	—	108
"	2.5	2	0	—	105
"	1.0	8	12	318	142
"	0.5	6	66	301	138
"	0.33	2	100	296	—
Glutathione	2.0	5	0	—	108
"	1.0	5	60	345	122
Thioglycolic acid	7.5	4	0	—	155
Alanine	7.5	4	100	368	—
NaCl	7.5	4	100	477	—
Phosphate buff					
pH 7.4	2.5	5	100	467	—
Ascorbic acid	4.0-7.5	4	100	610	—

effect of cysteine was due to the amino acid or the SH group

4 Ascorbic acid neutralized to pH 7.4 in doses of 4.0-7.5 mM/kg, in order to test the specificity of the SH reducing groups, as ascorbic acid is also a reducing agent

5 Thioglycolic acid, another sulfhydryl compound, was also used

The rats (Sprague-Dawley) were allowed excess food and water throughout the experiment. Blood sugars were determined by the Folin-Malmros micro method¹² on blood obtained by cutting the tip of the tail. Serial sugars were determined at 0, 3, 8, 24, 48 and, occasionally, at 72 hours. The rats were usually killed at 48 hours although a few were killed at 72 hours.

Data Following the injection of alloxan 3 phases of the blood sugar response are noted—an initial hyperglycemia, followed by a period of hypoglycemia, and finally, a late stage of permanent hyperglycemia or diabetes. As has been previously reported¹¹ animals injected intravenously with 40 mg/kg of alloxan usually reached their permanent hyperglycemic phase by 48 hours. Although, very occasionally, a late hypoglycemia occurred at 48 hours, these animals were also considered diabetic, for histologically the beta cells showed extensive necrosis. Ani-

mals were considered normal when the blood sugars at 48 hours were normal and all of the serial blood sugars were within the range of 90-160 mg %. In rats which were partially protected from the action of alloxan by inadequate doses of cysteine, slight initial hyperglycemic and hypoglycemic reactions were noted. The 48-hour sugars were normal or only slightly elevated but at 72 hours the sugars did not exceed 250 mg %. These animals were classed as mild diabetics. The tables list the average 48-hour blood sugars for the diabetic and normal animals and the 72-hour blood sugars for the mild diabetic animals. The biochemical findings presented in this paper correlate well with the cytological findings in the pancreas, which are reported in another paper¹³.

Reference to Table I shows that the intravenous injection of cysteine in doses of 2 mM/kg or greater, immediately before the administration of 40 mg of alloxan (equivalent to 25 mM/kg of alloxan) completely protected these animals against experimental diabetes. Cysteine dosages of 1 mM/kg protected most of the animals whereas with smaller doses nearly all the animals developed diabetes. When glutathione was used, complete protection was obtained when the intravenous dose was 2 mM/kg, but when glutathione was given in doses of 1 mM/kg

¹² Folin O and Malmros, H, *J Biol Chem*, 1929, 83: 115

¹³ Pridy, S L and Luzzatto, A, in press 1946

beta cells of the islets of Langerhans. Many workers have since confirmed these observations.

The mechanism of alloxan action is not understood. Since alloxan combines with sulfhydryl groups of protein⁴ and since many enzymes require sulfhydryl groups for their activity,^{5,6,7} it seemed possible that necrosis might result from enzyme inactivation by alloxan. Thus, it is noted that alloxan inhibits the activity of succinic dehydrogenase⁷ as well as the proteolytic enzymes, papain and cathepsin.⁶ Active sulfhydryl groups are also necessary for pyruvate oxidation and condensation, malate and ketoglutarate oxidation, d-amino acid and l-glutamic acid oxidation, and various fatty acid oxidations.⁷

Because many of the enzymes inactivated by alloxan can be reactivated, in part at least, by adding glutathione,⁷ a naturally occurring sulfhydryl compound, it was decided to test the effect of glutathione on the course of alloxan diabetes. Cysteine was used for part of the study because it is much cheaper than glutathione and because the sulfhydryl group of glutathione is contained in the cysteine component of the molecule.

In a preliminary note⁸ I reported that the injection of alloxan (200 mg/kg) intraperitoneally into rats immediately following an intravenous dose of neutralized cysteine or glutathione (7.5 mM/kg) did not cause diabetes. The injection of alanine, glycine, phosphate buffer, ascorbic acid, or succinic acid in place of the cysteine or glutathione did not protect the animals against alloxan. Leech and Bailey⁹ reported that the injection of 300-400 mg/kg of glutathione simultaneously with alloxan did not protect rabbits

from diabetes, and Weinglass *et al*¹⁰ reported that giving 300 mg/kg of alloxan subcutaneously simultaneously with 500 mg/kg of cysteine-HCl did not afford protection to rats. However, it is to be noted that the doses of cysteine and glutathione which I used were much greater than those used by other investigators, for 7.5 mM/kg is equivalent to 912 mg/kg of cysteine and 2,500 mg/kg of glutathione.

Methods In the preliminary studies alloxan was injected intraperitoneally. The intraperitoneal injection of alloxan into rats in doses of 200 mg/kg was not found to be reliable, for, although this dose usually produced diabetes in about 80% of the rats, occasionally an entire group of animals failed to respond. In contrast, intravenous injections of alloxan in doses of 40 mg/kg produced diabetes in 95% of the animals.¹¹ Therefore, this dose and route of administration were used in the present studies.

To facilitate the intravenous injection, vasodilation was induced by placing the rats in a 56°C oven for several minutes. A 2% solution of alloxan monohydrate was injected into the tail vein and blood was always drawn into the syringe before the injection was started. Solutions of cysteine hydrochloride or glutathione were carefully neutralized to pH 7.4 with NaOH and diluted so that each cc contained 0.2 to 0.75 mM. Usually one cc of solution was injected per 100 g of body weight. The cysteine was given either before or after the alloxan and different veins were used for each injection. As controls the following compounds were used and were injected in place of cysteine or glutathione:

1. NaCl (7.5 mM/kg), because NaCl is formed in the neutralization of cysteine-HCl.

2. Phosphate buffer, pH 7.4 (2.5 mM/kg), to rule out the effect of pH. Doses larger than 2.5 mM/kg caused a convulsive death, apparently due to hypocalcemic tetany.

3. Alanine, to test whether the protective

⁴ Lieben, F., and Edcl, L., *Biochem Z*, 1933 **259**, 8.

⁵ Hopkins, F. G., Morgan, E. S., and Lutwakh Mann, C., *Biochem J*, 1938, **32**, 1829.

⁶ Purp, A., *Biochem J*, 1935, **29**, 13.

⁷ Barron, E. S. G. and Singer, T. P., *J Biol Chem*, 1945, **157**, 221. Singer, T. P., and Barron, E. S. G., *J Biol Chem*, 1945, **157**, 241.

⁸ Lizarow, A., *Anat Rec*, 1945, **91**, 24.

⁹ Leech, R. S., and Bulev, C. C., *J Biol Chem*, 1945, **157**, 525.

¹⁰ Weinglass, A. R., Frame, E. G., and Williams, R. H. *Proc Soc Exp Biol and Med*, 1945, **58**, 216.

¹¹ Lizarow, A., and Palay, S. L., in press, 1946.

enzymes inactivated by alloxan

Alloxan can react directly with cysteine to form dialuric acid^{16,17} which is probably not diabetogenic^{17,18}. Bruckman and Wertheimer¹⁸ reported that a "super-saturated" solution of dialuric acid injected intravenously into rats did produce diabetes, but, since dialuric acid is rapidly converted to alloxan on standing in air,¹⁵ their results might be due to alloxan contamination.

Ascorbic acid is also reported as capable of reducing alloxan to dialuric acid.¹⁹ If the mechanism of sulfhydryl protection were simply reduction of alloxan to a nondiabetogenic compound dialuric acid one would also expect ascorbic acid to protect against alloxan diabetes. Ascorbic acid does not protect against alloxan diabetes, but rather appears to intensify the severity of the diabetes for the 48-hour blood sugars are considerably higher in the animals given ascorbic acid plus alloxan than are those of the rats given alloxan alone (610 mg per 100 cc compared with 397 mg per 100 cc). However interactions of ascorbic acid and alloxan within the body may not occur as readily as they do *in vitro*. Since alloxan exerts its diabetogenic effect within the first few minutes of injection,^{9,20} the relative rates of inter-reaction of alloxan with cysteine or with ascorbic acid might influence their protective abilities.

If the administered sulfhydryl groups were able to reactivate the enzymes inactivated by alloxan then giving the sulfhydryl compound after the administration of alloxan also should protect against diabetes. Leech and Bailey⁹ have shown that alloxan disappears from the blood within 2 minutes following its intravenous injection. Gomori and Goldner²⁰ found that when the vessels

to the pancreas were clamped before and for 5 minutes after the injection of alloxan then the beta cells in the clamped portion of the pancreas did not undergo necrosis. This fact indicates that the alloxan had disappeared from the blood during the period of clamping. However, in the present experiments the administration of cysteine one minute after alloxan produced only partial protection, whereas, when it was given after 3 minutes following the alloxan, no significant protection resulted. Therefore, if alloxan does inactivate the SH groups of enzymes, the administered glutathione or cysteine did not reactivate them after 3 minutes had elapsed. In this connection it is interesting that succinic dehydrogenase which has been inactivated by alloxan can be only partially reactivated by glutathione.⁵ The action of glutathione and cysteine, therefore, may consist of a protection of the sulfhydryl groups of the islet cells from inactivation as a consequence of the administration of large amounts of exogenous sulfhydryl compounds. Although this protection may simply be due to direct inactivation of alloxan by the administered SH groups, a more complex mechanism may also be involved.

The mechanism by which alloxan produces selective degeneration of the beta cells is not understood. Although other cells (kidney, liver, and occasionally, adrenal) may, at times, show degeneration following alloxan administration, the beta cells of the islets are by far the most sensitive to alloxan. Since the beta cells are highly specialized cells, for their main function apparently consists of the synthesis and secretion of insulin their enzyme systems are probably also highly specialized. In contrast the liver and kidney cells which do many things may have a number of alternate enzyme pathways. If the enzyme systems of the highly specialized beta cells require active sulfhydryl groups, whereas the less specialized cells of the body possessed alternate nonsulfhydryl pathways, the apparent selectivity of alloxan for the beta cells might be explained. However, this hypothesis could not easily explain the occasional occurrence of necrosis in the kidney or liver after the administration of

¹⁶ Lubes R, and Freisburger H, *Arch f exp Path u Pharm*, 1930, **156**, 226

¹⁷ Goldner, M G, and Gomori G, *Endocrinology*, 1944, **35**, 241

¹⁸ Bruckman, G, and Wertheimer E, *Nature*, 1945, **155**, 267

¹⁹ Ruben, J A, and Tipson, R S, *Science*, 1945, **101**, 536

²⁰ Gomori G, and Goldner M G, *Proc Soc Exp Biol and Med* 1945, **58**, 232

TABLE II
Protection Against Alloxan Diabetes: Cysteine 7.5 mM/kg Given Intravenously Either Before or After Alloxan 40 mg/kg I.V.

Substance given first	Time interval min.	No. animals	% diabetic		Blood sugars			
					48 hr severe diabetic	48 hr mild diabetic	72 hr mild diabetic	48 hr non diabetic
			Severe	Mild				
Cysteine	12	13	0	0	—	—	—	130
Alloxan	15	5	80	0	471	—	—	91
"	6	6	83	0	507	—	—	112
"	3	3	66	33	454	173	300	—
"	1	10	10	60	390	133	214	140

only some of the animals were protected. Thioglycolic acid in doses of 7.5 mM/kg was the only other agent tested which had any protective effect. Alanine, sodium chloride, phosphate buffer or ascorbic acid did not modify the course of alloxan diabetes.

Reference to Table II shows that when the alloxan was given prior to the sulfhydryl compound some protection against diabetes resulted if the time interval between the alloxan and the cysteine injections was small. If the interval was one minute, only one of 10 animals developed severe diabetes and 5 showed mild diabetes. Cytologically, however, all of these animals showed some evidence of pancreatic damage, although in 9 out of the 10 rats this consisted only of degranulation or necrosis of some of the beta cells in the islets. However, if the time interval between alloxan and cysteine was 3 minutes or longer, there was no significant protection against diabetes.

All of the cysteine-protected animals in this group were killed at the end of 48 or 72 hours for histological examination so that follow-up studies were not made. However, some of the rats reported in the preliminary note⁴ were followed for several months. These had received 7.5 mM/kg of cysteine intravenously immediately preceding 200 mg/kg of alloxan intraperitoneally. None ever did develop diabetes.[†]

[†] In 5 of 10 rats given alloxan i.p. and cysteine i.v., islets appeared on the 15th, 26th day. The rats suddenly began to gain weight rapidly and in some cases the abdominal cavities contained as much as 100 cc of fluid. The livers were shrunken and had a thickened capsule. Further studies are being carried out.

Discussion At pH 7.4 *in vitro* alloxan is rapidly converted to a non-diabetogenic compound, alloxanic acid.¹⁴ The fact that large doses of phosphate buffer (pH 7.4) injected intravenously just prior to the injection of alloxan did not protect animals against diabetes indicated that the cysteine effect is not due to any contained buffer. The fact that alanine did not protect against alloxan diabetes, whereas cysteine did, indicates that the protection is not due to a direct interaction between alloxan and amino acids—a reaction which is known to occur in the test tube.¹⁵ Since thioglycolic acid also protected against diabetes, the SH group appears to be responsible for the protective action of cysteine and glutathione.

Since the liver controls the addition of sugar to the blood, it was possible that normal blood sugars could have occurred in the presence of extensive necrosis of the islets, if the livers were also extensively damaged. However this was not the case for histological examination of the pancreas showed that the beta cells were normal when adequate doses of sulfhydryl compounds were given before alloxan.[†]

In explaining the protective effect of sulfhydryl groups one should consider the following possibilities:

1 Direct inactivation of alloxan by the SH group of glutathione or cysteine.

2 Protection of the sulfhydryl groups of enzymes from inactivation by alloxan.

3 Reactivation of sulfhydryl groups of

¹⁴ Richardson, G. M., and Cannon, R. K., *Biochem. J.* 1929, **23**, 68.

¹⁵ Archibald, R. M., *J. Biol. Chem.*, 1947, **158**, 347.

enzymes inactivated by alloxan

Alloxan can react directly with cysteine to form dialuric acid^{16,17} which is probably not diabetogenic^{17,18}. Bruckman and Wertheimer¹⁸ reported that a "super-saturated" solution of dialuric acid injected intravenously into rats did produce diabetes, but, since dialuric acid is rapidly converted to alloxan on standing in air,¹⁵ their results might be due to alloxan contamination.

Ascorbic acid is also reported as capable of reducing alloxan to dialuric acid¹⁹. If the mechanism of sulfhydryl protection were simply reduction of alloxan to a nondiabetogenic compound dialuric acid, one would also expect ascorbic acid to protect against alloxan diabetes. Ascorbic acid does not protect against alloxan diabetes, but rather appears to intensify the severity of the diabetes for the 48-hour blood sugars are considerably higher in the animals given ascorbic acid plus alloxan than are those of the rats given alloxan alone (610 mg per 100 cc compared with 397 mg per 100 cc). However, interactions of ascorbic acid and alloxan within the body may not occur as readily as they do *in vitro*. Since alloxan exerts its diabetogenic effect within the first few minutes of injection,^{9,20} the relative rates of inter-reaction of alloxan with cysteine or with ascorbic acid might influence their protective abilities.

If the administered sulfhydryl groups were able to reactivate the enzymes inactivated by alloxan, then giving the sulfhydryl compound after the administration of alloxan also should protect against diabetes. Leech and Bailey⁹ have shown that alloxan disappears from the blood within 2 minutes following its intravenous injection. Gomori and Goldner²⁰ found that when the vessels

to the pancreas were clamped before and for 5 minutes after the injection of alloxan then the beta cells in the clamped portion of the pancreas did not undergo necrosis. This fact indicates that the alloxan had disappeared from the blood during the period of clamping. However in the present experiments the administration of cysteine one minute after alloxan produced only partial protection, whereas when it was given after 3 minutes following the alloxan, no significant protection resulted. Therefore, if alloxan does inactivate the SH groups of enzymes, the administered glutathione or cysteine did not reactivate them after 3 minutes had elapsed. In this connection it is interesting that succinic dehydrogenase which has been inactivated by alloxan can be only partially reactivated by glutathione.⁵ The action of glutathione and cysteine therefore, may consist of a protection of the sulfhydryl groups of the islet cells from inactivation as a consequence of the administration of large amounts of exogenous sulfhydryl compounds. Although this protection may simply be due to direct inactivation of alloxan by the administered SH groups, a more complex mechanism may also be involved.

The mechanism by which alloxan produces selective degeneration of the beta cells is not understood. Although other cells (kidney, liver, and occasionally, adrenal) may, at times show degeneration following alloxan administration, the beta cells of the islets are by far the most sensitive to alloxan. Since the beta cells are highly specialized cells for their main function apparently consists of the synthesis and secretion of insulin their enzyme systems are probably also highly specialized. In contrast, the liver and kidney cells which do many things may have a number of alternate enzyme pathways. If the enzyme systems of the highly specialized beta cells require active sulfhydryl groups, whereas the less specialized cells of the body possessed alternate nonsulfhydryl pathways, the apparent selectivity of alloxan for the beta cells might be explained. However this hypothesis could not easily explain the occasional occurrence of necrosis in the kidney or liver after the administration of

¹⁶ Labes R, and Freisburger H. *Arch f exp Path u Pharm*, 1930, 156, 226

¹⁷ Goldner M G, and Gomori, G, *Endocrinology*, 1944, 35, 241

¹⁸ Bruckman, G, and Wertheimer L. *Nature*, 1945, 155, 267

¹⁹ Ruben I A, and Tipson R S. *Science* 1945, 101, 536

²⁰ Gomori G and Goldner M G. *Proc Soc Exp Biol and Med* 1945, 58, 232

TABLE II
Protection Against Alloxan Diabetes Cysteine 7.5 mM/kg Given Intravenously Either Before or After Alloxan 40 mg/kg I.V.

Substance given first	Time interval min	No animals	Blood sugars					
			% diabetic		48 hr severe diabetic	48 hr mild diabetic	72 hr mild diabetic	48 hr non diabetic
			Severe	Mild				
Cysteine	12	13	0	0	—	—	—	130
Alloxan	15	7	80	0	471	—	—	91
"	6	6	83	0	503	—	—	112
"	3	3	66	33	454	173	300	—
"	1	10	10	60	390	133	214	140

only some of the animals were protected. Thioglycolic acid in doses of 7.5 mM/kg was the only other agent tested which had any protective effect. Alanine, sodium chloride, phosphate buffer or ascorbic acid did not modify the course of alloxan diabetes.

Reference to Table II shows that when the alloxan was given prior to the sulfhydryl compound some protection against diabetes resulted if the time interval between the alloxan and the cysteine injections was small. If the interval was one minute, only one of 10 animals developed severe diabetes and 5 showed mild diabetes. Cytologically, however, all of these animals showed some evidence of pancreatic damage, although in 9 out of the 10 rats this consisted only of degranulation or necrosis of some of the beta cells in the islets. However, if the time interval between alloxan and cysteine was 3 minutes or longer, there was no significant protection against diabetes.

All of the cysteine-protected animals in this group were killed at the end of 48 or 72 hours for histological examination so that follow-up studies were not made. However, some of the rats reported in the preliminary note³ were followed for several months. These had received 7.5 mM/kg of cysteine intravenously immediately preceding 200 mg/kg of alloxan intraperitoneally. None ever did develop diabetes.[†]

[†] In 5 of 10 rats given alloxan 1 p.m. and cysteine 1 a.m. islets appeared on the 15th-28th day. The rats suddenly began to gain weight rapidly and in some cases the abdominal cavities contained as much as 100 cc of fluid. The livers were shrunken and had a thickened capsule. Further studies are being carried out.

Discussion At pH 7.4 *in vitro* alloxan is rapidly converted to a non-diabetogenic compound, alloxanic acid.¹⁴ The fact that large doses of phosphate buffer (pH 7.4) injected intravenously just prior to the injection of alloxan did not protect animals against diabetes indicated that the cysteine effect is not due to any contained buffer. The fact that alanine did not protect against alloxan diabetes, whereas cysteine did, indicates that the protection is not due to a direct interaction between alloxan and amino acids—a reaction which is known to occur in the test tube.¹⁵ Since thioglycolic acid also protected against diabetes, the SH group appears to be responsible for the protective action of cysteine and glutathione.

Since the liver controls the addition of sugar to the blood, it was possible that normal blood sugars could have occurred in the presence of extensive necrosis of the islets, if the livers were also extensively damaged. However, this was not the case for histological examination of the pancreas showed that the beta cells were normal when adequate doses of sulfhydryl compounds were given before alloxan.¹⁵

In explaining the protective effect of sulfhydryl groups one should consider the following possibilities:

- 1 Direct inactivation of alloxan by the SH group of glutathione or cysteine
- 2 Protection of the sulfhydryl groups of enzymes from inactivation by alloxan
- 3 Reactivation of sulfhydryl groups of

¹⁴ Richardson, G. M. and Cannan, R. K., *Biochem. J.*, 1929, **23**, 68.

¹⁵ Archibald, R. M., *J. Biol. Chem.* 1945, **158**, 347.

were studied by special granule stains, Gomori¹⁰ reported a decrease in the relative number of beta cells

3 Human beings do react to alloxan. Although it was first reported that man does not respond to alloxan, Brunschwig and Allen¹¹ have since reported that large doses of alloxan do produce an effect

4 Since alloxan occurs in some plants in its half-reduced form, alloxantin,¹² it might be ingested in the diet. This possibility becomes significant in the light of a report by Ruben and Yardumian¹² that oral administration of alloxan causes diabetes

5 The amount of alloxan actually necessary to produce diabetes is not large. For example, in the rat a small dose of 40 mg/kg produced diabetes consistently and 20 mg produced diabetes at times. Since the weight of the pancreas is less than 1% of the body weight, and since the beta cells constitute but a small fraction of the weight of the pancreas, the actual amount of alloxan necessary to destroy most of the beta cells in the rat is probably much less than 10 γ

Glutathione, normally present in the blood and tissues, may serve to protect the body

from any alloxan which might appear metabolically or in the diet. However, critical amounts of alloxan sufficient to injure the beta cells might conceivably appear within the human body under a number of circumstances.

a A metabolic defect resulting in excessive accumulation of alloxan in the body

b Decrease in glutathione concentration in the body resulting in a decrease in the normal protective mechanism against alloxan

c A specific metabolic defect of glutathione occurring only within the beta cells of the islets

From the evidence at hand it would be desirable to study the relation of alloxan and glutathione metabolism to the etiology of human diabetes

Summary 1 The intravenous injection of large doses of glutathione or cysteine, compounds which normally occur in the body, one to 2 minutes prior to the injection of a diabetogenic dose of alloxan completely protected rats from diabetes

2 Other amino acids, phosphate buffer, and ascorbic acid did not exert such a protective effect

3 When the sulfhydryl compounds were given one minute after the alloxan injection, partial protection occurred, whereas, when 3 or more minutes had elapsed, there was no protection

4 The mechanism and significance of this protection are discussed

¹⁰ Gomori, G, *Am J Path*, 1941, 17, 395

¹¹ Lippmann, L. O., *Ber*, 1896, 29, 2645, Rithausen, H., *Ber*, 1896, 29, 894, 2106, Fisher, H. I., and Johnson, T. B., *J Am Chem Soc*, 1932, 54, 2038

¹² Ruben, I. A., and Yardumian, K., *Am J Clin Path*, 1945, 15, 230

alloxan

Following the injection of alloxan into animals there is a rapid decrease in the glutathione content of the body. DeCaro and Rovida²¹ reported that within 10 minutes following the injection of alloxan into rats (200 mg/kg i.p.) the glutathione content of the liver fell to 17% while that of the intestine fell to 42% of their normal values. Leech and Bailey⁹ reported that following the injection of alloxan into rabbits (200 mg/kg i.v.) the blood glutathione dropped rapidly in some cases to almost zero. Within 5 minutes after the injection the blood glutathione values began to rise and returned to normal by 18 to 24 hours.

Because injected alloxan causes a decrease in the glutathione content of the tissues, and because glutathione protected against alloxan damage, it is possible that the amount of glutathione present within the cell may determine its resistance to alloxan. If the beta cells of the islets of Langerhans contained less glutathione than other cells of the body, their greater susceptibility to alloxan might thus be explained.

Various species have been reported to have different susceptibilities to alloxan. Hooded rats, for example, are more resistant to alloxan than are albino rats.²² Whereas a rat becomes diabetic after a dose of 40 mg/kg i.v., human beings can tolerate much larger doses without showing any effect.^{23, 24} However, a single dose of alloxan (600 mg/kg i.v.) did produce an effect on the blood sugar and islets of humans.²¹ Variations in species susceptibility to alloxan might also be explained, in part at least, by differences in the amount of glutathione or cysteine they contain.

The question of whether alloxan may be

involved in human diabetes is an interesting one.

1 Theoretically, at least, alloxan could be formed from uric acid, a normal metabolite. Under certain conditions an enzyme present in dog liver can convert uric acid into dialuric acid.²⁵ Dialuric acid is rapidly oxidized to alloxan by air. This latter reaction, however, may not occur as readily within the body, for the oxidation of dialuric acid to alloxan is inhibited by protein.²⁶ Studies of the possible formation of alloxan from uric acid within the human body need to be undertaken.

2 The changes seen in the pancreas of rats which have been given alloxan and have been diabetic for many months are not greatly different from those seen in human diabetes. Although some investigators have stated that the cytological changes of the pancreas in alloxan diabetes are quite different from those occurring in human diabetes, they have usually compared the lesions seen in human diabetics with those occurring in the acute stages following alloxan administration.²⁷ The islets of diabetic rats 5 to 7 months after the injection of alloxan appeared essentially normal when examined with the usual histological stains (hematoxylin and eosin). However, when they were examined by special granule stains, they showed a marked decrease in the number of beta cells present.²⁸ Similarly, the islets of human diabetics often appear normal when examined by the ordinary histological methods. It is doubtful whether the hyalinization seen in some cases of human diabetes is significant for it is not always present and may even be present in the absence of diabetes.²⁹ However, in 3 of 5 human diabetics whose islets

²¹ De Caro, L. and Rovida, E., *Boll. Soc. Ital. Biol. Sper.*, 1937, **12**, 611.

²² Duff, G. L., and Starr, H., *Proc. Soc. Exp. Biol. and Med.*, 1944, **57**, 280.

²³ Brunschwig, A., Goldner, M. G., Allen, J. G., and Gomori, G., *J. Am. Med. Assn.*, 1943, **122**, 966.

²⁴ Brunschwig, A., Allen, J. G., Owens, F. M., and Thornton, T. F., *J. Am. Med. Assn.*, 1944, **124**, 212; Brunschwig, A., and Allen, J. G., *Cancer Res.*, 1944, **4**, 45.

²⁵ Ascoli, M., and Izar, G. Z., *Z. physiol. Chem.*, 1909, **62**, 347; Prieti, L., *Z. physiol. Chem.*, 1909, **62**, 354.

²⁶ Dixon, M., and Zerfas, L. G., *Biochem. J.*, 1940, **36**, 371.

²⁷ Duff, G. L., *Am. J. Med. Sci.*, 1945, **210**, 381.

²⁸ Lazrow, A., and Palay, S. L., unpublished observations.

²⁹ Warren, S., *The Pathology of Diabetes Mellitus*, 2nd ed., Lea and Febiger, Philadelphia, 1938.

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²⁵ Ascoli, M., and Izor, G. Z., *J. physiol. Chem.*, 1909, **62**, 347; Pictet, L., *J. physiol. Chem.*, 1909, **62**, 354.

²⁶ Dixon, M., and Zerkis, L. G., *Biochem. J.*, 1940, **36**, 371.

²⁷ Duff, G. L., *Am. J. Med. Sci.*, 1945, **210**, 381.

²⁸ Larsson, A., and Pictet, S. L., unpublished observations.

²⁹ Wrenn, S., *The Pathology of Diabetes Mellitus*, 2nd ed., Lea and Febiger, Philadelphia, 1938.

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